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ON THE TOXIC AND ALLERGIC MANIFESTATIONS PRODUCED BY THE TISSUES AND FLUIDS OF ASCARIS. I. EFFECT OF DIFFERENT TISSUES

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The purpose of this paper is to discuss the evidence as to whether the toxic effects often attributed to the tissues and fluids of ascaris should be regarded as due to a direct pharmacological action, to the presence of an antigenic toxin, or to the phenomenon of hypersensitivity, and to present some new observations on this subject.

a. *Evidence for a direct toxic effect of ascaris tissues.*—Many descriptions have appeared in the literature of toxic effects following the administration of ascaris substances to animals. Some investigators describe symptoms resembling anaphylactic shock following intravenous injection of ascaris tissues, especially the body fluid (Schwartz, 1923 and Simonin, 1920). Others have observed digestive disturbances and degenerative changes in the visceral organs following oral administration of ascaris body fluid and the saline in which these worms have been living (Iio, quoted by Girges, 1934; Sakaguchi, 1928; and Read, 1931). Herrick and Emery (1929) found that ascaris extracts caused an increase in tonus and rate of contraction in the isolated intestine of several mammals and confirmed the finding that extracts given per os caused vomiting and diarrhea. Observations cited thus far were made following the administration of crude preparations of ascaris tissues and fluids, and chemical changes may have occurred consequent upon protein disintegration. More recently, it has been observed (see Discussion) that ascaris extracts from which protein and glycogen had been removed caused shock in guinea pigs and dogs following intravenous injection. On the other hand, Ransom, Harrison and Couch (1924) were unable to elicit toxic effects in laboratory animals by administering ascaris tissues by any parenteral route. Vanni (1938) injected filtered body fluid into guinea pigs and rabbits by various routes and found it to be completely harmless even in large doses. Eisenbrandt (1942) injected chickens subcutaneously with saline extracts of ascaris in 4 doses, each containing 12, 14, 31 and 56 mg protein respectively, but no harmful effects were described.

b. *Evidence for the presence of a true toxin in ascaris.*—Taliaferro (1929) cites two observations in which the investigators considered a true toxin to be present in ascaris: Shimamura and Fujii (1917) recovered what they called an "albumose-peptone" from aqueous extracts of *Ascaris lumbricoides* and *Parascaris equorum*. This fraction was toxic to horses, guinea pigs, dogs and rabbits, but not to mice and rats. These workers observed that horses could be "immunized" by sublethal amounts injected intravenously and that they became less susceptible although no antitoxin was demonstrated. Weinberg and Julien

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(1911a and b, and 1913) found that the body fluid of *P. equorum* when instilled into the eye of the horse caused, in many instances, acute conjunctivitis and edema of the eyelids. Julien (1926) attempted to show that the reaction was caused by a toxin against which the horse could be immunized as a result of infection. These two reports do not, as Taliaferro points out, prove that a toxin and antitoxin are involved since no antitoxin was demonstrated. The results obtained by Weinberg and Julien were probably manifestations of hypersensitivity, and the toxic factor in the experiments of Shimamura and Fujii may have been similar to that studied by Macheboeuf and Mandoul (1939). Disappearance of the toxic effect and its absence in some infected animals may have been a result of desensitization.

c. *The sensitizing properties of ascaris tissues.*—Tissues and fluids of ascaris are known to incite hypersensitivity in certain animals. Manifestations of the allergic state are evident in some individuals infected with ascaris (Coventry and Taliaferro, 1928), in individuals who work with the worm (Jones and Kingscote, 1935), and in animals experimentally sensitized with ascaris substance (Dukelskaia and Golubeva, 1927; Coventry, 1929; and Moraes, 1932). The mechanisms involved in ascaris sensitization, however, are unknown, and it is likely that many animals may have an unsuspected sensitization. Sensitization to ascaris protein may arise from infection not only with ascaris but also with other nematodes, especially with those belonging to the orders Ascaroidea, Trichuroidea and Oxyuroidea (Maternowska, 1934 and Schonfeld, 1937). Ransom, Harrison and Couch (1924) observed that certain pigs, sheep and dogs have positive skin tests and conjunctival reactions with ascaris antigens. Brunner, Altmann and Bowman (1944) obtained positive skin tests in 12 out of 25 dogs, and from the observations of Weinberg and Julien (see above) horses may exhibit ascaris allergy. Since it is not known whether ascaris sensitization is attributable to nematode infection, to exposure to ascaris antigens other than by infection or to what extent sensitization may be influenced by heredity, reports of the toxicity of ascaris substances in which hypersensitivity is not definitely excluded must be interpreted with caution. As Taliaferro (1929) pointed out, the proteins of the parasite may be relatively harmless to the normal host but may become highly toxic owing to sensitization.

Antibodies are involved in the mechanism of ascaris allergy because, although they cannot always be demonstrated by precipitin tests (Rackemann and Stevens, 1927 and Jadassohn, 1928), serum of a sensitized person will transfer sensitivity when injected into a normal person or animal either locally at the site of injection into the skin (Prausnitz-Kustner reaction) or generally when injected intraperitoneally (passive anaphylaxis). Thus, Rackemann and Stevens (1927), Jadassohn (1928), Coventry and Taliaferro (1928), Brunner (1934) and Tezner (1934) have shown that the serum of a person sensitized to ascaris conveys a local skin sensitivity to a normal person. Brunner, Altmann and Bowman (1944) have shown that this is also true in dogs. Ciculesco-Mavromati (1927) sensitized a guinea pig by the serum of a man infected with ascaris. Oliver-González (1946) reported that guinea pigs injected intraperitoneally with the serum from rabbits immunized with whole worm material showed anaphylactic symptoms when they were injected intravenously with the intestine or body fluid of the worm 48 hours later but not

with cuticle, muscle, egg or sperm. He observed a similar sensitization to the body fluid and intestine in guinea pigs experimentally infected 8 days previously.

Ascaris extracts, therefore, are capable of eliciting allergic reactions but there is doubt as to whether they contain directly toxic substances active in the non-sensitized animal. Three factors may be responsible for this confusion: First, experimental animals may be unsuspectedly hypersensitive, either through present or past infection with various nematodes or by exposure to unknown antigens; second, extracts have varied from crude saline extracts to purified protein-free extracts; and third, various methods of administration have been used.

The following experiments were designed to test the effect of different tissues and fluids of ascaris by different routes of administration to sensitize and non-sensitized animals. The worm tissues were prepared in such a way as to minimize chemical changes.

METHODS

Ascaris ovary, body fluid, cuticle, intestine, muscle, and whole worm were obtained by dissecting living *A. lumbricoides* from the pig. They were immediately frozen at −76 C, lyophilized and stored in vacuo (Sprent and Chen, 1949). Stored lyophilized materials were used during the course of two to three months. One percent suspensions of these tissues, finely ground, were made in 0.85% saline, were kept for one hour at 37 C and overnight in the refrigerator. For intraperitoneal injection the whole suspension was used; for intravenous injection only the supernatant fluid was used. Saline in which worms had been living for 12 hours was also frozen, lyophilized and diluted in distilled water.

EXPERIMENTAL

a. *Direct effect. Experiment 1.*—Each of 6 different ascaris suspensions was administered as follows: to two 3,000 g rabbits in doses of 5 cc intraperitoneally, repeated 4 times at 4-day intervals; to one 3,500 g rabbit in a single intravenous dose of 5 cc; to two 400 g guinea pigs in a single intraperitoneal dose of 3 cc; to one guinea pig in a single intravenous dose of 2 cc; to one mouse in a single intravenous dose of 0.5 cc; and to 6 mice in intraperitoneal doses of 0.5 cc, repeated 4 times at 4-day intervals. This experiment involved 78 animals, and in no instance was there any symptomatic indication that the injection caused any harmful effect. The dried saline in which worms had been living for 12 hours did not affect a rabbit given 4 intraperitoneal doses of 5 cc at 4-day intervals or 4 mice given 0.5 cc similarly. Five guinea pigs showed no symptoms following the oral administration of 2 cc of 1% body fluid repeated on three successive days.

Experiment 2.—Sections of liver, lungs, heart and kidney of a series of mice (see exp. 7), each of which had been injected intraperitoneally 4 times with one of the different ascaris tissues, were examined for degenerative changes. No abnormal changes were evident in the myocardium or kidneys, but the cytoplasm of the liver cells of some of the mice were vacuolated and their nuclei pyknotic (table 1). In all mice there was an increased periportal infiltration of lymphocytes, and in those injected with cuticle, body fluid, intestine and whole worm, a considerable number of heterophil and eosinophil leucocytes were scattered among the lymphocytes (fig. 1). In the lungs the changes were uniform and striking and consisted of a thickening of the alveolar wall, mobilization of the septal cells, and an infiltration of lymphocytes and, in many cases, eosinophils (fig. 2).

Experiment 3.—To investigate further the liver changes caused by ascaris tissues, mice were injected intraperitoneally with 0.5 cc of 1% ascaris body fluid and their livers preserved at the times shown in table 2. In those mice killed

one to two days after the last injection (15, 16, 17, 21 and 22), degenerative changes were evident in the liver cells. In those mice killed 11 days after the third dose of body fluid (18, 19 and 20), degenerative changes were not conspicuous, although the liver appeared to have been damaged previously. These findings indicate that the liver cells are damaged by the injected fluid, while the incidence of periportal infiltration suggests that this phenomenon is associated with immunization.

TABLE 1.—*Immunological and histological† findings in mice injected with ascaris tissues (intraperitoneal injections).*

Number of mice injected	Tissue injected	Precipitin titer	Periportal infiltration (with lymphocytes) in liver	Periportal infiltration (with granulocytes) in liver	Degenerative changes in liver	Alveolar infiltration and proliferation of septal cells in lungs
3	Cuticle	1:12,800	+	+	−	+
2	Body fluid	1:12,800	+	*	−	+
2	Whole worm	1,6,400	+	+	+	+
2	Intestine	1:3,200	+	+	+	+
2	Muscle	not tested	+	−	+	+
2	Ovary	not tested	+	−	+	+

* One mouse showed a ± and the other a + granulocytic infiltration.
† No degenerative changes occurred in the kidney and in the heart, and skin tests were all negative.

b. *Active sensitization. Experiment 4.*—Six guinea pigs were given a sensitizing intraperitoneal dose of 3 cc of 1% suspension of each ascaris tissue including whole worm, and 16 days later were given an intravenous dose of 0.1 cc of the supernatant fluid (see methods) from the same tissue. All the animals showed anaphylactic shock.

Experiment 5.—Each of 6 mice was given an intravenous injection of 0.5 cc of the different tissue suspensions, followed by a second similar injection after a 12 day interval. No anaphylaxis occurred.

Experiment 6.—The intradermal injection of 0.1 cc of the supernatant fluid from the ascaris tissue suspensions did not cause any skin reaction in normal rabbits. After intraperitoneal injections of 5 cc of 1 % tissue suspensions, however, each tissue suspension when injected intradermally, either undiluted or diluted 5 times, gave rise to a skin sensitivity at the times indicated in figure 3. Figure 3 also shows the precipitin titer of the serum as indicated by ring tests with dilutions of the homologous antigen.

Experiment 7.—Each of the 6 ascaris tissue suspensions in 0.5 cc amounts was injected intraperitoneally into three mice 56, 38, 29 and 7 days before the mice were killed. Twenty-four hours before death each mouse received an intradermal injection of 0.1 cc of the homologous tissue suspension. No skin reactions were observed with any of the tissues although the mice had developed precipitins (table 1).

The three foregoing experiments demonstrate that in animals which are most susceptible to hypersensitivity (rabbits and guinea pigs), sensitizing properties were manifested by all tis-

TABLE 2.—*Effect of intraperitoneal injections of ascaris body fluid (0.5 cc of 1% solution in saline) on the liver of mice.*

Mouse	Number of daily injections*	Time killed after start of experiment (days)	Liver changes	
			Degenerative changes	Periportal infiltration
15	1	2	+	−
16	2	3	+	−
17	3	4	+	−
18	3	14	−	+
19	3	14	−	±
20	3	14	−	−
21	4	14	+	+
22	4	14	+	+

* Injections were on first three consecutive days except no. 21 and 22, whose 4th injections were on the 12th day.

sue suspensions. This conclusion is corroborated by my own experience. During the first year of working with ascaris the worms were often handled and the dried powders inhaled without ill effect. About a year after the work was started a transient irritating rash was observed on the hands when the worms were handled. Severe nasal symptoms began

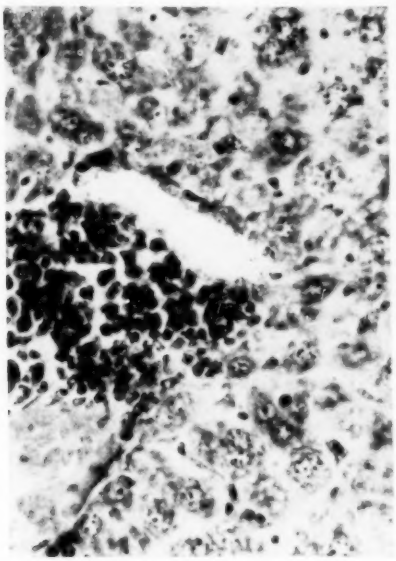

FIG. 1.—Liver of mouse injected 4 times intraperitoneally with 0.5 cc of ascaris intestine (1% suspension).

to develop when dried ascaris tissues were inhaled and became more severe as time went on. The nasal symptoms were followed by asthmatic breathing, which would pass off but return again at night with increased severity.

A series of skin tests performed at the time hypersensitivity had developed are illustrated in figure 4. No infection with ascaris had taken place. Another uninfected individual and an individual recently infected with ascaris were tested in the same way but both were negative.

FIG. 2.—Lung of mouse injected 4 times intraperitoneally with 0.5 cc of ascaris body fluid (1% suspension).

During this time my serum gave no positive precipitin reactions to any ascaris materials.

c. *Passive sensitization. Experiment 8.*—All ascaris rabbit antiserums except whole worm antiserum obtained from the rabbits whose skin reactions and precipitin titers are depicted in figure 3 caused passive sensitization of guinea pigs to anaphylaxis 24 hours after injection of the homologous antigen. Five controls with a preliminary injection of normal serum elicited no symptoms.

DISCUSSION

In discussing these results together with the findings of previous workers, the toxic effects of ascaris must be considered in two categories:

1. Toxic effects resulting from the previous sensitization of the experimental animal.

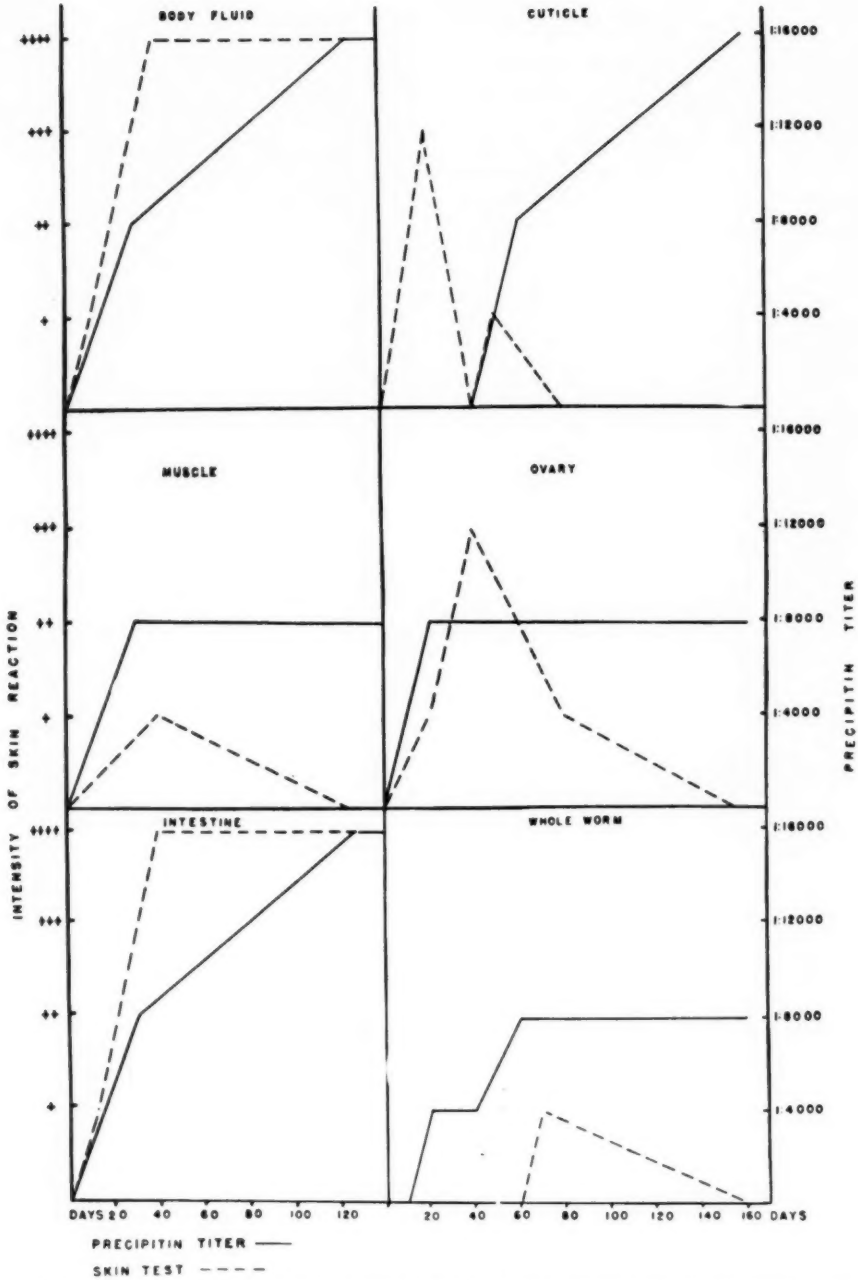

FIG. 3.—Skin reactions and precipitin titers of rabbits injected intraperitoneally with ascaris antigens at 5, 12, 18, 115, 120 and 125 days.

2. Toxic effects resulting from pharmacologically active substances in the worm.

There is no doubt that all ascaris tissues are potent sensitizing agents, and Brunner (1934) has stated that ascaris extracts appear to have some characteristic which makes them unusually potent atopens. Some reports of the toxicity of whole ascaris extracts may, therefore, have been the result of using animals with an unsuspected hypersensitivity. This applies particularly to observations on horses and carnivores, which may harbor nematodes of the family Ascaridae. Animals may also become sensitized in other ways than by infection.

Concerning the second category, experiments in this paper indicate that no symptoms followed administration of ascaris tissues if anaphylactic conditions were avoided, but that liver cells were damaged and infiltration of cells into the periportal and peribronchial tissues were evident. The infiltrations were probably a result of immunization rather than a direct effect.

Reports of anaphylacoid symptoms following administration of crude ascaris extracts in cases where previous sensitization was unlikely may have been due to protein disintegration and the formation of toxic amines such as histamine. Werle (1936) has shown that certain mammalian tissues contain decarboxylases which convert histidine into histamine. Similar decarboxylation may occur in ascaris extracts if they are not immediately lyophilized. For example, Schwartz (1923) used *Ascaris vitulorum* after removal from calves following treatment with turpentine. As some of these worms may have been dead, partial disintegration of their proteins may have occurred. Furthermore, Herrick and Emery (1929) found that extracts prepared from dried worms produced a more potent effect on isolated mammalian intestine than those prepared from fresh worms. The effective agent may have been produced during the drying process, which was of relatively long duration. The effects obtained were not, however, identical to those produced by histamine, although Deschiens and Poirier (1947) have demonstrated the presence of histidine or

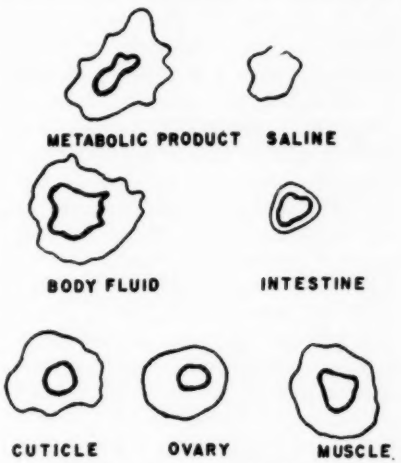

FIG. 4.—Skin reactions on the arm of a person sensitized by exposure in the laboratory to *Ascaris lumbricoides*. A 1% suspension of each antigen was scratched into the skin. The drawing was made 30 minutes later. The thin line demarcates the area of redness; the thick line demarcates the area of the wheal. (One-half natural size.)

histamine in protein-free ascaris extracts by chemical test (vide infra).

Other phenomena which may explain some of the reports of the toxic effects are the reactions of Schwartzman and of Sanarelli. Vanni (1938) observed that while the body fluid of ascaris is harmless in normal rabbits (see Introduction), it sometimes (15%) produces a local Schwartzman reaction accompanied by profound shock, desquamation of the intestinal mucosa and renal damage.

Protein-free ascaris extracts evidently contain a substance which on intravenous injection causes a fall in blood pressure and symptoms of anaphylactic shock. This was demonstrated in the dog by Emery and Herrick (1929) from *A. lumbricoides*, and the albumose-peptone used by Shimamura and Fujii (1917) was probably a similar substance. Macheboeuf and Mandoul (1939) believe such a substance from extracts of *P. equorum* to be a polypeptide. Mandoul (1939) demonstrated that the substance is not an antigen because it will not cause sensitization in sublethal doses. Rocha e Silva et al. (1946) believe that a similar substance from *A. lumbricoides* is a proteose and found that it caused profound and fatal shock in the dog, accompanied by engorgement of the liver and increase in blood histamine and heparin. The symptoms and physiological changes indicate very strongly that the toxic effects involve a physiological change identical to that occurring in true anaphylactic shock, possibly consisting of a constriction of the hepatic veins. Deschiens and Poirier (1947) confirmed that the extract caused shock in the guinea pig. They demonstrated the presence of histamine or histidine in the extract by the Pauly diazo reaction and were able to prevent its toxic effect by previous injection of the animal with antihistamine drugs, although the extract would not cause a contraction of the isolated intestine of the guinea pig.

In discussing the anaphylactic effect of ascaris extracts on dogs, Rocha e Silva et al. (1946) incline to the view that ascaris extracts are toxic because the host has been previously sensitized by various intestinal helminths. Accordingly, the only evidence supporting the primary toxic effect of ascaris extracts is that given by the observations made on guinea pigs, which often do not harbor nematodes.

The present state of our knowledge indicates that all ascaris tissues and fluids contain potent anaphylactogenic and allergenic substances. While dilute suspensions are not toxic in mice, guinea pigs and rabbits which have not been previously sensitized, there is evidence to show that certain substances derived from proteins such as albumose-peptones (Shimamura and Fujii, 1917), polypeptides (Macheboeuf and Mandoul, 1939) and proteoses (Rocha e Silva et al., 1946) may be extracted which cause anaphylactic symptoms. But it has not been definitely proved that this will occur in animals which have never been previously sensitized. The presence of histamine itself in ascaris extracts has not been demonstrated biologically. There is no conclusive evidence to show that toxic substances are excreted into the bowel of the host by any of the ascarid worms.

SUMMARY

Whole worms and isolated ascaris tissues were frozen, lyophilized and stored in vacuo. Repeated intraperitoneal or single intravenous injections of these materials failed to produce toxic symptoms in rabbits, guinea pigs and mice. The body fluid caused no symptoms when given orally to guinea pigs. Repeated injections led to degenerative changes in the hepatic cells, increased periportal cellular infiltration, and cellular infiltration of the aveolar septa. These substances were shown to cause active anaphylactic sensitization of the guinea pig to the same antigen, skin sensitivity and precipitins in rabbits and a skin reaction in a person sensitized to ascaris, although the serum of this person showed no evidence of precipitins against any of the antigens. In mice the

tissues and body fluid caused the production of precipitins but no sensitivity as tested by active anaphylaxis and skin tests. Rabbit antiserums against ascaris ovary, cuticle, muscle, body fluid and intestine caused passive anaphylactic sensitization in the guinea pig against the homologous antigen.

The sensitizing potency of the ascaris substances and the lack of anaphylactoid effects following single intravenous injections of the ascaris materials preserved in such a way as to reduce protein breakdown to a minimum, make it probable that the toxic effects simulating anaphylaxis previously described by several investigators were in some cases due to (1) unsuspected hypersensitivity in the experimental animals, or (2) toxic substances occurring in the extracts as a result of the disintegration of protein. It is evident that peptone-like substances whose effect has been demonstrated in extracts freed of certain proteins by trichloracetic acid are present in the body of ascaris, but whether they are active in the absence of previous sensitization has not been conclusively established.

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VIRUS ISOLATION AND SEROLOGIC STUDIES ON PATIENTS WITH CLINICAL MUMPS

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The etiologic agent of mumps was demonstrated in 1934 when a typical parotitis was produced in Rhesus monkeys with filtrates of saliva from mumps patients.[1] The discovery in 1945 that mumps virus would multiply in the developing chick embryo[2] has provided a more convenient host for detailed study of the virus. As a result, rapid advances have been made in knowledge concerning the properties of the virus and in the development of laboratory methods for its identification.

Several recent reports have described the isolation in chick embryos of mumps virus from human material. Beveridge, Lind and Anderson[3] recovered virus from 4 of 12 specimens of human saliva by yolk sac inoculation. Leymaster and Ward[4] isolated virus from the saliva of 8 of 9 patients with clinical mumps by amniotic inoculation. Reports concerning the isolation of mumps virus from the spinal fluid of patients with meningoencephalitis are not numerous. Mawson and Swan[5] were successful in isolating virus from the spinal fluid of one patient by inoculation of monkeys. Henle and McDougall[6] have recently reported the isolation of mumps virus from two patients by amniotic inoculation of cerebrospinal fluid into chick embryos.

An epidemic of mumps which occurred in Atlanta during the winter of 1947–48 presented an opportunity for further studies on serologic tests and for the isolation of virus from patients with various manifestations of mumps. The results are reported in the present paper.

METHODS

Isolation of virus.—Saliva and saline washings of the mouth were collected from patients within 1 to 7 days after the onset of parotitis. In addition, cotton swabs were held over the orifices of Stenson's ducts for several minutes, then placed in the washings previously obtained. About one-fourth volume of tryptose phosphate broth was added to all materials. Cerebrospinal fluid was obtained from patients with meningoencephalitis as soon after admission to the hospital as possible. Hydrocele fluid was obtained from two patients with orchitis at the time of orchidotomy by inserting a needle through the parietal layer of the sac of the tunica vaginalis and aspirating with a syringe. A small piece of testicular tissue was removed from one of the patients following incision of the tunica albuginea. This was ground with alundum and suspended in tryptose phosphate broth. All materials not inoculated immediately into eggs were stored at −20 C until used.

The above materials were inoculated into the amniotic sac of 8-day-old embryonated hens' eggs. A small square area was inscribed over the air sac with a dental drill, and this area of shell was removed. Inoculations were made in a dark room with the eggs on a candle box. The embryo usually can be seen by this method without wetting the shell membrane. The inoculating needle was gently thrust through the membrane toward the embryo and 0.2 ml injected into the amniotic sac. A metal cap was paraffin-sealed over the hole in the shell and the eggs incubated at 37 C for 5 to 6 days. Penicillin and streptomycin were routinely added to all materials so that each egg received 200 units of each antibiotic. Six to 10 eggs were inoculated with each material.

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At the end of the incubation period the eggs were iced overnight at 4 C and the amniotic fluids harvested individually. Each amniotic fluid was tested for agglutination of chicken red blood cells. Identification of the infective agent as mumps virus was performed on all fluids containing hemagglutinins by means of the complement fixation and the hemagglutination-inhibition tests. Serums from patients recovering from mumps were used in the complement fixation test, and high titer antiserums prepared in chickens against mumps virus were used in the inhibition test. Fluids giving negative hemagglutination reactions initially were pooled and carried through two subsequent passages in embryonated eggs before being considered negative.

Serologic tests. a. *Agglutination inhibition.*—Serial two-fold dilutions of serum in 0.25 ml volumes were mixed with an equal amount of virus suspension containing a known concentration of hemagglutinin. A 0.6% suspension of chicken red blood cells was then added to each tube in 0.5 ml amounts. The final concentration of hemagglutinin in each tube was 2 units. After one hour at room temperature the results were read by observing the presence or absence of the characteristic pattern of agglutinated cells. The titers are expressed as the reciprocal of the highest dilution of serum producing complete inhibition.

b. *Complement fixation.*—Dilutions of serum were mixed with one and one-half units of complement and an amniotic fluid suspension of mumps virus in dilution of 1:2. Each material was used in a volume of 0.2 ml. The unit of complement was determined by a preliminary titration in the presence of the amniotic fluid used in the test. In addition to the usual anti-complementary control, normal amniotic fluid in dilution of 1:2 was included in each test. After incubation at 37 C for one hour, 0.6 ml of a 3% suspension of sensitized sheep red cells was added to each tube. The test was read after an additional 30 minutes at 37 C and the titer expressed as the reciprocal of the highest dilution of serum giving a two-plus or better fixation.

RESULTS

Isolation of virus from patients

1. *Saliva and mouth washings.*—Mouth washings were obtained from 17 patients with parotitis within three days after the onset of salivary gland involvement and tested for virus as described under Methods. Virus was obtained from 10 of these specimens (58.8%). Usually the amniotic fluids from only one or two eggs of the first passage showed unequivocal hemagglutination. A second amniotic passage, however, usually yielded fluid with hemagglutinin titers up to 1:128. Two passages were usually adequate to establish the presence of virus in the specimens. No positive results were obtained by repeated amniotic passages of those fluids which were negative on the initial passage. Virus was not recovered from mouth washings taken from three patients 7 days after onset of parotitis.

TABLE 1.—*Isolation of mumps virus in chick embryos.*

Manifestation	Material studied*	Number of patients	Number yielding mumps virus
Parotitis	Saliva	17	10
	Blood	3	0
	Placenta	1	0
Meningo-encephalitis	Spinal fluid	7	0
Orchitis	Hydrocele fluid	2	0
	Testicular extract	1	1

* Material from patients with parotitis was obtained within three days after onset of illness; from patients with meningocephalitis, 4 to 10 days; and from patients with orchitis, 6 days after onset.

2. *Spinal fluid.*—Seven patients with meningoencephalitis were studied, all of whom had parotitis which began from 4 to 10 days before the onset of encephalitic symptoms. The spinal fluid in each instance showed a lymphocytic reaction with cell counts ranging from 195 to 620 cells per cmm. Tests for virus in the spinal fluids by amniotic inoculation in chick embryos were completely negative.

The clinical diagnosis of mumps meningoencephalitis was confirmed by serologic studies. These patients were usually seen late in the course of their illness, i.e., 4 to 10 days after the onset of parotitis. It was not unusual for the first serum specimen taken on admission to show a hemagglutination inhibition titer of 1:64 or higher or a complement

fixation titer of 1:32. A four-fold or greater rise in titer, however, occurred in each case during convalescence. Also of interest was the detection of antibodies in the spinal fluid drawn from one patient on admission, 7 days after the onset of parotitis.

Acute and convalescent serums collected from 12 patients with lymphocytic meningitis of unknown etiology during the six-month period preceding the outbreak of the mumps epidemic were also tested for mumps complement fixing antibodies with negative results. During the mumps epidemic, however, most of the cases of this type of meningitis were shown to be due to infection with mumps virus.

3. *Hydrocele fluid and testicular tissues.*—Although it is supposed that invasion of the testicle by mumps virus is responsible for the frequently occurring orchitis, isolation of virus from this organ has not been previously reported. Two patients with mumps orchitis were studied. Attempts to isolate virus from the hydrocele fluid of both patients failed, but mumps virus was isolated from testicular tissue obtained from one of the patients. A brief summary of the latter case follows:

R. B., An 18-year-old male, was admitted to the hospital on February 16, 1948, for treatment of orchitis. Six days before admission the patient developed pain and swelling in the right parotid region, which subsided uneventfully after about 4 days. Tenderness of the right testicle developed two days before admission, and 24 hours later it became acutely painful and markedly enlarged. On admission the patient's temperature was 104 F. There was slight swelling in the right parotid area. The right testicle was markedly enlarged and extremely tender to palpation. The remainder of the physical examination was non-contributory.

Soon after admission right orchidotomy was done. Hydrocele fluid and testicular tissue were obtained as described under Methods. Following operation the patient's temperature rapidly returned to normal, and pain and swelling in the right testicle subsided. Pathologic examination of the testicular tissue revealed swollen but well-preserved seminiferous tubules and a minimal infiltration of the interstitial tissues by large mononuclear cells.

Acute serum collected on admission fixed complement in a dilution of 1:32 as compared to a titer of 1:128 obtained with serum drawn 6 weeks later. Virus was not isolated from saliva collected on admission, i.e., 6 days after onset of parotitis, or from hydrocele fluid obtained at operation. In the first and second amniotic passages of the testicular extract, however, a slight degree of hemagglutination was observed with some of the fluids. In the third passage unequivocal hemagglutination was observed. The virus was well established by the 4th passage, the pooled amniotic fluid from this passage having a hemagglutination titer of 1:256.

4. *Blood.*—Defibrinated blood was collected from three patients during the first or second day after the onset of parotitis. No virus was detected on inoculation into chick embryos.

5. *Placenta.*—Of particular interest was a pregnant patient who developed mumps parotitis 5 days prior to delivery. The baby was isolated immediately after delivery and did not develop any signs of illness. An attempt to recover virus from an extract of the placenta was unsuccessful. Acute and convalescent serums obtained from the baby failed to show antibodies by either agglutination inhibition or complement fixation tests, although the convalescent serum of the mother contained mumps antibodies in high titer. No evidence of placental transfer of virus, therefore, was obtained in this instance.

Comparison of serologic titers with three mumps viruses

Acute and convalescent serums from 15 patients with uncomplicated parotitis were studied by means of the hemagglutination inhibition and complement fixation tests. Three viruses were compared in these tests. One of the viruses was obtained from Dr. J. F. Enders and has been maintained by amniotic pas-

sages in this laboratory. It is designated as I for purposes of presentation. Two viruses isolated from patients in the present work were also tested. One (II) was recovered from the saliva of a patient with parotitis, and the other (III) was isolated from the testicular tissue of a patient with orchitis. The latter viruses were in their 5th to 8th passages when used in the serologic tests.

The results of these tests are summarized in table 2. Most of the acute serums had hemagglutination inhibition titers of 1:32 or less and complement fixation titers of less than 1:8. The convalescent serums of all of the patients contained significantly higher titers of agglutination-inhibiting and complement-fixing antibodies against the three test viruses. Furthermore, it will be seen that there was no significant difference in the titers obtained with the three viruses. In general, the antibody response as measured by the inhibition test was similar to that obtained with the complement fixation test.

No major difference in the antigenic structure of the three viruses was apparent, therefore, in these tests. Further serologic tests with antiserums prepared in chickens against these viruses likewise failed to reveal any strain differences. One difference, however, which has also been observed by other workers[6] is that the recently isolated strains (II and III) multiply only in the amniotic sac, while the stock strain (I) propagates in both amniotic and allantoic sacs.

TABLE 2.—*Serologic tests on acute and convalescent serums from patients with mumps parotitis.*

Patient no.	Agglutination inhibition			Complement fixation		
	Test virus†			Test virus		
	I	II	III	I	II	III
1	<32–256	<32–128	32–256	<8–128	<8–256	<8–64
2	<32–64	<32–64	<32–512	<8–32	<8–64	<8–128
3	<32–128	<32–64	<32–64	<8–512	16–128	<8–128
4	32–512	<32–256	<32–128	<8–256	<8–256	8–256
5	32–256	<32–64	32–256	<8–256	<8–256	16–1024
6	32–1024	<32–1024	<32–256	<8–256	<8–512	<8–128
7	32–128	<32–128	32–256	<8–64	16–128	<8–64
8	<32–512	<32–256	64–128	<8–128	<8–64	<8–256
9	64–256	32–512	64–128	<8–64	<8–128	<8–128
10	<32–64	32–128	<32–256	<8–128	<8–128	<8–128
11	32–512	<32–256	<32–256	<8–64	<8–128	<8–64
12	<32–128	<32–64	<32–256	<8–64	<8–256	<8–64
13	<32–64	32–128	<32–128	<8–128	<8–128	<8–128
14	32–256	<32–256	<32–64	<8–64	<8–128	8–128
15	<32–64	32–128	32–128	<8–32	<8–32	<8–64

* First number in each column represents acute serum titer; the second number, the convalescent serum titer. Titers are expressed as reciprocal of the dilution end point (see Methods).
† See text for source of virus.

parotitis yielded virus by amniotic inoculation in the chick embryo. The testicular tissue of a patient with mumps orchitis also yielded virus by this method. Virus was not demonstrated in (a) the saliva of three patients taken 7 days after the onset of parotitis, (b) the spinal fluid of 7 patients with meningoencephalitis, (c) hydrocele fluid of two patients with orchitis, (d) blood of three patients taken during the first or second day of illness, and (e) a placental extract from a patient with parotitis.

Henle and McDougall[6] reported the isolation of mumps virus from the spinal fluid of two patients by amniotic inoculation in chick embryos. It should be noted that both of these patients had meningoencephalitis without preceding

DISCUSSION AND SUMMARY

In the experiments herein reported, 58% of mouth washings obtained within three days after the onset of mumps

parotitis and the spinal fluids were obtained within two days after the onset of illness. In the present study the patients with meningoencephalitis had salivary gland involvement from 4 to 10 days prior to the onset of symptoms referable to the central nervous system. It seems likely, therefore, that the failure to recover virus from the spinal fluids of these patients might be attributable to antibodies which had formed by the time of appearance of meningoencephalitis. Actually, antibodies were demonstrated in the spinal fluid of one patient and it was not unusual to find circulating antibodies at this time as reported by Enders.[7]

Positive serologic tests were obtained in all of the patients studied by both the hemagglutination inhibition and complement fixation tests. Although comparable rises in titer were observed with the two methods, the complement fixation test was more reliable and gave more reproducible results. On several occasions a preparation of virus, although containing a high titer of hemagglutinins, reacted poorly in the inhibition test. The same virus suspension, however, gave satisfactory results in the complement fixation test.

No strain differences could be detected between two mumps viruses isolated in the present epidemic and a virus isolated by Dr. J. F. Enders several years ago. It is of interest that the striking and readily demonstrable strain differences observed with influenza virus, which has many properties in common with mumps virus, have not been encountered thus far with mumps virus.

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FURTHER STUDIES ON B5W, AN ANAEROGENIC GROUP IN THE ENTEROBACTERIACEAE

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In 1935 an unknown organism was isolated in almost pure culture from fecal specimens of each member of a family of 4 with acute diarrhea. Large numbers of the same organism were isolated from a small portion of adequately refrigerated three day old milk suspected of causing the gastroenteritis. These organisms were short gram negative nonsporeforming nonmotile rods. They produced acid in glucose but failed to ferment lactose, sucrose, salicin, maltose or mannitol, failed to produce indole, acetylmethylcarbinol or hydrogen sulphide and grew readily on citrate agar. An antiserum was prepared from one of the unknown strains. Agglutination and adsorption tests showed that the strains from the 5 sources were antigenically identical. A limited search of the laboratory records and the literature on enteric bacteria failed to disclose previous isolations or descriptions of this organism. The antiserum and its homologous culture were placed in stock.

In 1937 the same organism was isolated from 9 patients and personnel involved in a gastroenteritis epidemic in a small infirmary. The source of the epidemic was not determined. These 9 strains agglutinated to a low titer in the antiserum prepared from the strain isolated in the previous epidemic. An intensive search of the literature revealed that the unknown organism resembled some of the cultures isolated from stored feces by Parr and Galbraith (1937) and called by them the "P bacillus," after Clemesha (1912). Between 1937 and 1943 a number of strains of the "P bacillus" were isolated from fecal or urine specimens of patients hospitalized with gastroenteritis or urinary infections. Since early in this period the culture isolated in the first epidemic and the antiserum prepared from it were discarded, no serological work was done on the new isolates.

In 1944, while investigating the serological relationships of coliform and related bacteria from infants in two unassociated institutions, 10 strains of the "P bacillus" were isolated from one nstitution and none from the other (Stuart and van Stratum, 1945). These new strains and the fact that several hundred fecal specimens from normal students in college and nursing schools failed to yield the "P bacillus" aroused new interest in the organism. An antiserum prepared from one of the new strains showed that the 10 strains were serologically identical. These 10 strains were isolated on SS (Shigella-Salmonella) agar, whereas all previous strains were isolated on EMB (eosin methylene blue) agar. The new strains appeared to differ from earlier ones by fermenting lactose in 2 to 3 weeks, but how long the carbohydrate tests were incubated in much of the previous work is not known. For reasons not pertinent to the present work the designation "P bacillus" for these organisms was changed to B5W (a white colony isolated from the 5th

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specimen from institution B). A program to continue investigating these organisms had to be abandoned at this time.

In 1947–48 work on the B5W organisms was resumed. In approximately one year 55 strains were collected from hospital and public health laboratories as far south as Florida and as far west as Illinois. Where the sources of the cultures were given, they seemed to be divided equally between feces and urine. Two strains were isolated from the blood stream. To date well over 100 strains of B5W have been encountered in this laboratory. All strains were anaerogenic and nonmotile. All cultures grew equally well on standard, EMB and desoxycholate lactose agar plates, with colonies ranging from 2 to 4 mm. Colonies on EMB agar varied markedly in character. No growth was observed on bismuth sulphite agar in 24 hours, but black colonies from 0.5 to 1.0 mm. appeared in two or three days. A few strains grew well on SS agar; many strains yielded very small (0.1 to 0.2 mm) colonies in one or two days, while a few strains failed to grow on this medium. All strains fermented glucose, arabinose and xylose rapidly. The fermentation of glucose was quite characteristic; acid was formed rapidly in the medium outside the vial while three or more days were required to give an acid reaction inside the vial using bromcresol purple indicator. A majority of the strains fermented rhamnose in 10 to 35 days. Lactose was attacked slowly by some strains and not at all by others. Sucrose, salicin, maltose, mannitol, adonitol, dulcitol and inositol were not fermented. The urease test in highly buffered medium (Rustigian and Stuart, 1941) was negative, but in weakly buffered medium a few cultures were positive. Indole, acetylmethylcarbinol and hydrogen sulphide were not produced. All strains grew readily on citrate agar (table 1). The methyl-red test was unusual. A great majority of the strains were negative but a few were weakly positive. The use of different hydrogen ion indicators showed that in the presence of the buffer in the Difco MR-VP medium the p_H seldom dropped below 6.0 to 5.8 in several days.

TABLE 1.—*Biochemical reactions of the nonmotile B5W group and a possible variant.*

No. strains	Glucose	Lactose	Sucrose	Salicin	Maltose	Mannitol	Arabinose	Xylose	Rhamnose	Adonitol	Dulcitol	Inositol	Indole	Methyl Red	Voges-Proskauer	Citrate	Hydrogen Sulphide	Urea	Motility
3	A	–	–	–	–	–	A	A	–	–	–	–	–	–	–	+	–	–	–
22	A	–	–	–	–	–	A	A	A^s	–	–	–	–	–	–	+	–	–	–
9	A	A	–	–	–	–	A	A	–	–	–	–	–	–	–	+	–	–	–
21	A	A^s	–	–	–	–	A	A	A^s	–	–	–	–	–	–	+	–	–	–
26	A	A^s	–	–	–	–	A^s	A	A^s	–	–	–	–	–	–	+	–	–	+

A =acid.
A^s =acid produced slowly.
A^s =acid produced slowly by some strains and not at all by others.

Concomitant with the work on B5W, the use of 10% lactose agar slants recommended by Chilton and Fulton (1946) to increase the speed of lactose fermentation was being studied. The agar base was prepared and sterilized. When sufficiently cool, Berkefield filtered lactose was added. The mixture was tubed aseptically and slanted. The reactions of 465 *Paracolobactrum*, *Proteus*, *Salmonella*, *Shigella* and unidentified cultures which ferment glucose rapidly

and lactose slowly or not at all were compared on the 10% lactose agar and in 1% lactose broth. All B5W strains gave a positive reaction on the 10% lactose agar in 16 to 24 hours. However, 1% lactose agar slants were almost as satisfactory as 10% lactose agar slants in speed of reaction. On these mediums no difference in the speed of fermentation could be detected between strains fermenting 1% lactose broth slowly and those failing to ferment in any time up to 90 days. A number of anaerogenic cultures more or less resembling B5W and a few *Proteus* cultures, negative in 1% lactose broth, gave a positive reaction in 3 to 9 days on 10% lactose agar. No significant differences were noted in the speed of reaction of other cultures, especially aerogenic strains, in the two mediums. The value of 10% lactose agar slants seemed limited to anaerogenic cultures or to cultures producing low gas volumes.

Antiserums were prepared from strains A267 and R222. Another antiserum, B5W, prepared in 1944 was available. Agglutination and adsorption tests confirmed a previous assumption that the B5W group was antigenically heterogeneous. Twenty-six strains and 5 strains (not counting the strains homologous to the antiserums involved) were antigenically identical to A267 and R222, respectively. A group of 6 strains agglutinated to high titers in both A267 and R222 antiserums but not at all in B5W. Upon adsorption of A267 and R222 antiserums with one of the 6 strains, agglutinins for the other 5 strains were completely removed. Therefore these 6 strains could have been antigenically identical or closely related. For the same reasons another group of 6 strains agglutinating in A267, R222 and B5W antiserums could have been identical or closely related. Ten strains either failed to agglutinate in any antiserum or agglutinated at titers too low to warrant drawing any conclusions regarding their relationship. There was no serological agreement between cultures fermenting and failing to ferment lactose and between cultures positive and negative in rhamnose. The 12 strains not fermenting rhamnose belonged to 4 different serological groups.

The antigenic behavior of many of the B5W group was peculiar. Living antigens agglutinated to titers of 5120 to 20480 after incubating two hours at 37 C and overnight at 55 C. Instead of the expected compact, granular O-type of agglutination at 55 C, soft masses of adherent cells were observed on the bottom of the tubes. Upon shaking, these masses disintegrated into short threads (Stuart et al., 1948). Antigens heated in a boiling water bath for one hour became almost completely inagglutinable, while another portion of the same antigens heated to 121 C for $2\frac{1}{2}$ hours agglutinated with a characteristic O reaction to the same titers as living antigens. It is hoped that this aspect of the B5W group can be investigated further.

Representative strains of the B5W group were tested in 54 antiserums prepared against serologically different anaerogenic cultures, including all generally accepted *Shigella* species and types, many Boyd and Sachs types and some undesignated cultures. In not a single instance was a significant reaction obtained with either living or heated antigens.

The limitations of the B5W group are not easily defined. During the one year period of the present investigation 26 strains from Florida and one from Rhode Island, except for being motile and fermenting arabinose slowly, possessed the same biochemical reactions as B5W. These motile strains attacked 10% lactose agar slants in two to three days. The motile strains failed to ag-

glutinate in the B5W antiserums, and an antiserum prepared from a motile strain failed to agglutinate the B5W strains. This complete lack of antigenic relationship between motile and nonmotile strains is not surprising, since only two motile strains possessed any antigens in common with the motile strain used to produce the antiserum. Both the nonmotile and motile forms were isolated from one patient.

DISCUSSION

Apparently there are no promulgated rules to determine how a new bacterial species, group or type (exclusive of antigenic type) should be reported in the literature. The mere number of strains and/or the time involved do not seem to be satisfactory criteria. In this laboratory 25 or more strains of a new organism have been isolated from a restricted area on one occasion but not encoutered again. A new organism has been isolated from a single institution over a two or three year period, after which it completely disappeared. Over a period of several years only 8 to 10 strains of a new organism have been collected from widely separated areas. While these new organisms were isolated from patients with gastroenteritis, in no instance did it seem advisable to give specific designation, much less species rank, to the organisms in question.

Within the last ten years several hundred strains of a new organism were isolated from widely separated areas, including three continents. This organism was given specific designation, 29911 (Stuart et al., 1946). Species rank seemed inadvisable since within the present taxonomic framework there was no satisfactory genus for this organism. Biochemically and antigenically the 29911 group appears to be a transitional form between *Proteus* and *Shigella*.

Cultures of B5W also appear to belong to a transitional group. They can be easily differentiated from other anaerogenic species and genera of the Enterobacteriaceae by their ability to give a rapid positive reaction on agar slants containing filtered lactose. They differ from *Proteus rettgeri* in their failure to give a positive reaction in highly buffered urea medium and in their inability to produce indole or ferment sucrose and mannitol. Unlike type 29911, they ferment xylose and arabinose rapidly but do not produce indole or attack sucrose. The property of utilizing citrate readily distinguishes B5W cultures from *Shigella;* moreover the lack of antigenic relationship between the B5W group and all generally accepted *Shigella* would tend to eliminate them from this genus.

Borman et al. (1944) advocated a "proshigella" genus for anaerogenic species which for various reasons did not warrant inclusion in the genus *Shigella*. The 29911 and B5W groups would fall naturally into a proshigella genus or its equivalent. Judging from work in the laboratories of Brown University and the Connecticut State Department of Health on more than 2,000 gram negative, nonsporeforming, anaerogenic cultures isolated from feces and urine, there is a relatively large number of these species or groups. Perhaps the time has come to put some limitations on giving specific designation or species rank in the family Enterobacteriaceae such as a minimum number of strains isolated from different areas over a period of time varying with the number of strains and areas concerned.

A review of the cases from which B5W strains were isolated in this laboratory or local hospital and public health laboratories would indicate that this group possessed a pathogenicity comparable to the less restricted *Shigella*, 29911 and

a number of unidentified anaerogenic organisms. Personal communications from other laboratories, however, describe some severe infections with high fever and isolations of B5W from the blood stream. The pathogenicity of these organisms probably varies within wide limits with high virulence infrequently encountered.

CONCLUSIONS

A new group of organisms isolated from feces and urine of, for the most part, hospitalized patients is described and given specific designation, B5W. Eosin methylene blue and desoxycholate lactose agar are satisfactory mediums for the isolation of B5W, while Shigella-Salmonella and bismuth sulphite agar are not. The group appears to be antigenically heterogeneous and to vary markedly in pathogenicity.

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FURTHER OBSERVATIONS OF AN INHIBITOR IN HUMAN SERUMS OF THE HYALURONIDASE PRODUCED BY A STRAIN OF HEMOLYTIC STREPTOCOCCUS

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In a previous report[1] an inhibitor of the hyaluronidase produced by a strain of *Streptococcus hemolyticus* of group A, type 4 was shown to be present in many human serums. There was a tendency for the titer of this inhibitor to be higher in serums obtained during convalescence from streptococcal infections and in individuals with rheumatic fever. No inhibitory effect against streptococcal hyaluronidase was possessed by normal serums from several species of laboratory animals. No human serums showed any inhibition of the hyaluronidase in testicular extracts. Serums with high titers of inhibitor of streptoccocal hyaluronidase had no comparable titer against hyaluronidase from *Staphylococcus aureus* or *Diplococcus pneumoniae;* the inhibitor was apparently specific for the hyaluronidase of *Strep. hemolyticus.* Quinn[2,3] has recently confirmed the finding of higher titers of inhibitor of streptococcal hyaluronidase in serum from individuals with rheumatic fever and streptococcal infections.

A number of reports[4,5,6,7,8] have also appeared recently dealing with hyaluronidase inhibitors in normal animal and human serums. There are some differences immediately apparent between the inhibitors with which these reports are concerned and the one under study here, but the information regarding both has been insufficient to make a clear distinction. The present report is an attempt to further elucidate this problem and obtain further information regarding the nature of the inhibitor.

PART I. THE INHIBITORY TITERS IN SERUM FROM INDIVIDUALS OF DIFFERENT AGES

Methods

Serums were obtained from a group of individuals between the ages of 2 weeks and 10 years.*

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* The author wishes to thank Dr. Paul B. Beesom of the Emory University School of Medicine, whose kind cooperation made possible the collection of these serums in the wards and clinics of the Grady Hospital, Atlanta, Georgia.

These individuals had come to the hospital for a variety of reasons, but none was suffering from a disease of streptococcal etiology when seen, nor did any give a history of such a disease in recent months. It was presumed that all except the very young members of the group may have had streptococcal infections sometime in the past. These serums were tested for their ability to inhibit the hyaluronidase of a strain of group A, type 4, hemolytic streptococcus. The mucin clot prevention test was performed as previously described.[1]

TABLE 1.—*Inhibitory titers in serum from individuals of different ages.*

Inhibitory titers	Age				
	Month		Years		
	0–3	3–12	2–4	5–7	8–10
3		3	2	1	
9		2	1	1	
27		1	2		
81	1		3		2
243	5		2	5	6
729	2			1	2
Number tested	8	6	10	8	10

Results

The results of the test are presented in table 1. It is apparent that serum from individuals younger than 3 months had relatively high inhibitory titers, comparable with those found in serum of members of the older age group. Individuals 3 to 12 months old had relatively low inhibitory titers as compared with those observed in the younger or older age groups.

Discussion

Antibodies produced as a consequence of widely prevalent human infections have a characteristic distribution in the serums of individuals of different ages. Such antibodies are present in at least a low titer in the serum of most adults. Most of these antibodies are transferred across the placenta into the fetal circulation, and serums from very young infants therefore usually contain antibody in amounts comparable to that in adult serums. After about three months most of this passively acquired antibody is lost, and until that individual becomes infected and actively produces antibody of his own, his serum is devoid of it. After one year of age the proportion of individuals with antibody present increases.

Streptococcal infections are almost universal in man, and each individual probably undergoes a number of them during a lifetime. Antibodies to streptococcal antigens should conform to the distribution of such a universal antibody with placental transfer. The inhibitor under study follows such a pattern.

PART II. OTHER STUDIES OF THE INHIBITOR OF STREPTOCOCCAL HYALURONIDASE IN HUMAN SERUM

Methods

The mucin clot prevention test was the method of hyaluronidase assay used. Certain modifications of the technique used in Part I and in previous work[1] were thought necessary in order to attain sufficient accuracy to evaluate changes in the inhibitory titer in serial serum specimens from one individual. These modifications are described below. The timing of the test, the actual steps involved, and the volumes of the various ingredients were not altered from the method used previously.

Preparation of the enzyme.—Brain heart infusion medium (Difco) was prepared and 0.2% potassium hyaluronate was added before sterilizing.* One thousand cc of this medium was inoculated with 1 cc of a broth culture of a streptococcus. After overnight growth the culture was centrifuged. The supernatant was removed and divided into 20 cc portions which were stored at −70 C until used as enzyme. Lots of enzyme were prepared from streptococci of group A, types 4 and 22, and groups C and G.† The enzyme solu-

* It was found that without added hyaluronate occasional lots of enzyme had little or no activity and that this variability was prevented by the addition of 0.2% hyaluronate to the culture medium. The addition of hyaluronate to this medium did not cause the great enhancement of enzyme production that has been reported upon addition of hyaluronate to synthetic mediums.[9]

† The type 4 strain was that used previously. The strain of type 22 was B242. The group C and G strains were C74 and D176B.

9. Rogers, H. J. 1945, Conditions controlling

tions used in the tests prevented formation of the mucin clot at a maximum dilution of 1:250.

Composition of the substrate.—The substrate used contained three parts 0.05 *M* McIlwaine's buffer at p_H 6.8 and one part 0.15% potassium hyaluronate. The hyaluronate was prepared from human umbilical cords by the method described by McLean et al.[10] A single lot was used for testing all of the serums reported here. The 0.15% solution of hyaluronate was prepared and stored at 4 C over chloroform until used. Substrate was prepared in amounts sufficient for from one to three days work and stored in a similar fashion. One cc was used in each tube in the test.*

Reagent for formation of the mucin clot.—In the previous report the serum required for formation of the mucin clot was incorporated in the substrate, and 2 *N* acetic acid was added at the end of the test. Since the substrate used here contained no serum, it was necessary to add a solution containing 10% normal horse serum and 2 *N* acetic acid to test for clot formation at the end of the procedure. Two-tenths cc of this "acid serum reagent"[11] was added to each tube.

Storage of serums and preparation for testing.—The serums had been stored at 4 C for about 8 months before being received at this laboratory. From that time until they were tested they were stored at −70 C. The serums were all heated at

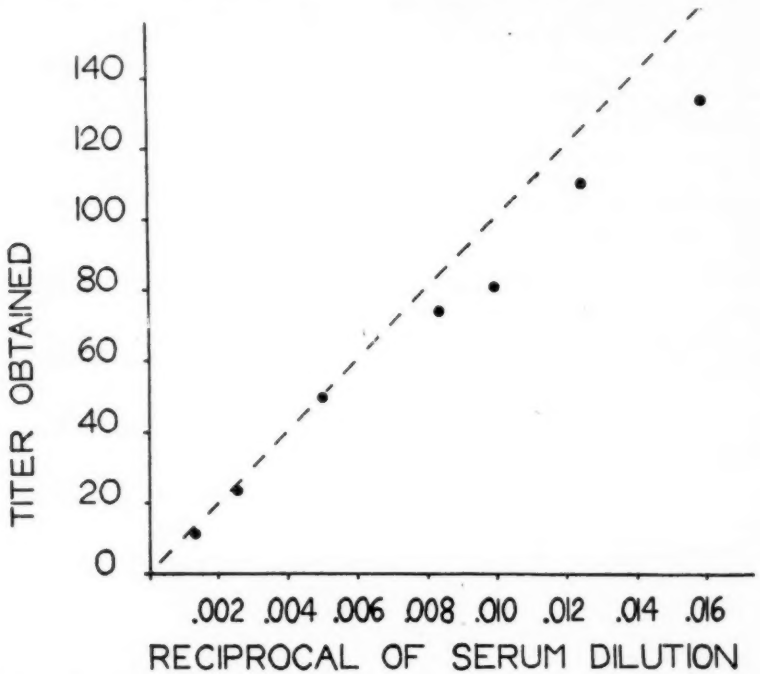

FIG. 1.—The effect of variation in amount of hyaluronidase inhibitor of the titer obtained. The amount of inhibitor was varied by testing different dilutions of the same serum specimen.

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10. McClean, D., Rogers, H. and Williams, B. 1943, Early diagnosis of wound infection. Lancet **1:** 355–360.

* The substrate had a lower salt concentration than that used previously. The decreased enzyme activity attained with this substrate seemed to be due to this difference.

11. Pike, R. 1946, A study of group A streptococci from healthy carriers with particular attention to mucoid polysaccharide production. J. Jnfect. Dis. **79:** 148–165.

56 C for 30 minutes before being tested, with the exceptions noted below. After the period of heating a 1:200 dilution of each serum in distilled water was prepared for the test. All serum specimens from each individual were tested simultaneously in the preliminary test.

Definition of units of enzyme and inhibitor.—One-half cc of the highest dilution of the stock enzyme solution which prevented formation of the clot was considered to contain one unit of enzyme. One unit of inhibitor was defined as the amount which would inhibit no more than one unit of enzyme. Each serum was tested against a series of tubes containing amounts of enzyme which varied by increments of 20%. A preliminary test was carried out against amounts of enzyme in the middle of the test range, and then against a lower or higher range if no end point was obtained in the first test. The inhibitory titer of a serum represents the largest number of units of enzyme completely inhibited by that serum.

For such a titer system to be valid, it is essential that the titer obtained should reflect the actual amount of inhibitor present, i.e., should be in direct proportion to it. That this is so, within the limitations of the test, is shown in figure 1. Here the amount of inhibitor present has been varied by testing different dilutions of one serum. The reciprocal of the dilution of serum being tested expresses the relative concentration of inhibitor at each point. Although this figure represents the results of tests on one serum only, others have been tested and found to conform to these results.

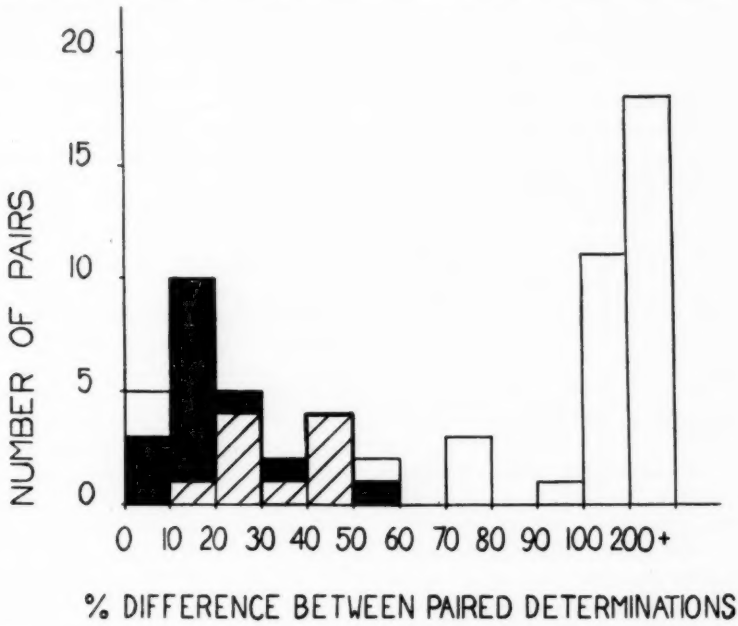

FIG. 2.—The changes in titer of inhibitor of streptococcal hyaluronidase during scarlet fever. The white columns show the distribution of the 50 cases studied according to the percent difference between the initial titer and the maximum titer reached in each. Repeat determinations were done on 25 serums and the range of the percent variation within each pair is shown in black to indicate the extent of the experimental error in the test method. Crosshatching is used to show where black and white areas overlap.

Reading the test.—A clot develops in tubes if they contain no enzyme, if enzyme is neutralized by antiserum, or if enzyme is diluted beyond detection. Tubes near the end point usually show partial disintegration of the clot and occasionally only a few threads. A tube is not considered positive for the enzyme unless there are no threads or

shreds remaining. Turbidity alone or a fine homogeneous precipitate in a tube is read as positive.

Estimated accuracy of the test.—During the testing of the serums, repeat determinations were run on 25 individual serum specimens. The results of these are shown in the black columns in figure 2. Here the difference, if any, between the members of the pairs is expressed as the percent of the smaller of the two. In no case was this difference more than 60%.† On the basis of this figure a change in titer of 70% or more in any individual case was considered to be due to some cause other than experimental error.

The effect of heating at 56 C on the inhibitory titer of serums

Several serums were heated at 56 C to test the stability of the inhibitor under such conditions. The inhibitory titers of a 1:200 dilution of the heated serums and an unheated sample of the same serum specimens were determined simultaneously. The results are given in table 2. It can be seen that none of the titers of the serums decreased measurably in 30 minutes at that temperature and that only after two hours did any measurable decrease in titer occur.

TABLE 2.—*The effect of heating at 56 C on the inhibitory titer.*

Period at heating at 56 C	Serum				Pooled human γ globulin, 1:400 dilution
	1	2	3	4	
Unheated	29	50	74	55	158
15 minutes					158
30 minutes	29	50	74	55	132*
1 hour				55	72
2 hours				55	
3 hours				45	
4 hours				38	

* When the gamma globulin solution is heated for longer than 15 minutes a precipitate forms. This point was not investigated further.

Changes in titer of hyaluronidase inhibitor during scarlet fever and rheumatic fever

A. Clinical material studied.—In the spring of 1946 a detailed study of 375 cases of scarlet fever was made by the personnel of the Naval Medical Research Unit Number 4 and the Rockefeller Institute for Medical Research at the Great Lakes Naval Training Center, Great Lakes, Illinois.* Frequent notes were made of clinical observations, electrocardiograms, and erythrocyte sedimentation rates. Throat cultures and bleedings for serum were done on admission and at weekly intervals. The streptococci isolated were types 1, 3, 17, 19, and 30. All hemolytic streptococci isolated were identified according to serological group and type.† Serum was stored at 4 C for later use. One serological study on these cases has already been reported by Anderson, Kunkel and McCarty,[12] and detailed studies of the clinical features are under preparation by the personnel of the Naval Medical Research Unit Number 4.[13]

Fifty cases from this series were studied. These were divided into three groups according to the clinical and laboratory features of their disease. Group 1 contained 21 cases of uncomplicated scarlet fever. Group 3 con-

† When serums were titered simultaneously the titers did not vary by more than one tube (20%).

* The author is very much indebted to those who, as members of the staff of the Naval Medical Research Unit Number 4 in 1946 and later, carried out the clinical studies on these cases. He also wishes to express his appreciation to Dr. Rebecca C. Lancefield through whose kindness the serum from these patients and the cultures of streptococcal strains tested for hyaluronidase production were made available.

† All of the strains of group A hemolytic streptococcus isolated from these patients were tested for production of hyaluronidase by the mucin clot prevention test. The results of these tests were uniformly negative. The significance of this finding is to be discussed.

12. Anderson, H. A., Kunkel, H. and McCarty, M. 1948, Quantitative antistreptokinase studies in patients infected with group A hemolytic streptococci: A comparison with serum antistreptolysin and gamma globulin levels with special reference to the occurrence of rheumatic fever. J. Clin. Investigation **27:** 425–434.
13. Studies of the Naval Medical Research Unit Number Four. To be published.

sisted of 13 cases of scarlet fever followed by typical rheumatic fever. The requirements for inclusion of a case in this group were the occurrence during the period under study of arthritis or arthralgia, increased erythrocyte sedimentation rate or fever, and an abnormal electrocardiogram.* Group 2 consisted of 16 cases showing one or two of these findings. The patients studied were all males between 17 and 21 years of age.

Serum was not available at each week in every case. In 8 cases only 3 bleedings were tested. In the remainder 4 or 5 were available. The bleedings were spread over a period of at least 20 days in all of the cases. In 7 cases the period studied was less than 28 days; in 43 cases, between 28 and 45 days. All available serums were tested against the hyaluronidase from a strain of *Strep. hemolyticus* of group A, type 4.

Of the 50 cases studied, 22 received salicylates at one time or another during their illness. This was almost always given in a divided daily dose of 9 g of sodium salicylate accompanied by 3.6 g of sodium bicarbonate. Serum salicylate levels were not determined. Serums obtained during periods of salicylate administration are indicated by italics in table 3.

B. Results.—

1. Changes in titer during scarlet fever and convalescence.—The results of the tests for inhibition of the type 4 enzyme are presented in table 3. The mean titer for all the serums obtained in the week of onset of the disease was 27, but there was a range around this point from 1 to 82. A similar great variation in the titer of inhibitor was present at all of the weekly intervals. A comparison of the titers at the time of the first bleeding and at the maximum in each case is of interest. The initial titer was 30 or lower in 70% of the cases, while the maximum titer reached was greater than 30 in 80%. It is apparent that there was a general upward trend following the onset of the infection.

A consideration of the percent change in titer in the individual cases is more revealing because it does not exclude those starting relatively high but still showing a marked rise in titer. An increase in titer of 70% over the initial value was considered to be a significant rise. Because of the high dilution at which the serums were tested, such an increase in titer was accepted as indicating a definite rise even if the actual maximum value reached was not very high. Only three cases had a rise in titer of more than 70% without reaching a maximum value of 15 or greater. There was no appreciable difference between groups 1, 2 and 3, and they were therefore all considered together. In figure 2 the white columns show the cases grouped according to the percent rise in titer each showed. A rise of more than 70% took place in 33 cases (66%), more than 100% in 30 cases (60%), and more than 200% in 18 cases (36%). Two individuals showed no change in titer. Nine had one or more bleedings with a lower titer than the first. Only one of these showed a decrease in titer of more than 70%. In 6 of the 9 showing a decrease, the downward change was 20% or less.

2. Time required to reach a significant rise in titer.—In the 33 cases showing a significant rise, this point was not usually reached very early in the disease. Figure 3 shows the number of cases reaching a titer 70% or more above the initial value in each two

* Abnormalities considered significant were T wave abnormalities in the limb leads according to the New York Heart Association criteria, appearance of 1st degree heart block, or an abrupt increase in the P-R interval of .06 second or more in the absence of prolongation beyond .20 second.

TABLE 3.—*Titers of hyaluronidase inhibitor in 50 cases of scarlet fever.*

Group 1. Uncomplicated scarlet fever								Group 2. Questionable rheumatic fever								Group 3. Rheumatic fever							
Case	Enzyme		Week of disease					Case	Enzyme		Week of disease					Case	Enzyme		Week of disease				
	Group	Type	0	1	2	3	4–6		Group	Type	0	1	2	3	4–6		Group	Type	0	1	2	3	4–6
6	A	4	—	17	54	54	45	20	A	4	24	28	—	59	*50*	13*	A	4	67	67	100	—	*120*
		22	—	6	27	27	19			22	14	16	—	30	*90*			22	61	61	73	—	*100*
	C	—	—	3	3	3	0		C	—	3	3	—	*3*	*0*		C	—	1	6	6	—	*6*
	G	—	—	0	0	0	0		G	—	0	0	—	0	*0*		G	—	2	2	5	—	*4*
10	A	4	8	—	18	13	10	66	A	4	1	—	11	13	11	181*	A	4	21	31	*61*	*50*	*45*
		22	7	—	11	7	9			22	4	—	11	19	19			22	19	30	*43*	*36*	*36*
	C	—	0	—	3	0	0		C	—	3	—	0	0	0		C	—	1	1	*3*	*1*	*1*
	G	—	0	—	0	0	0		G	—	0	—	0	0	2		G	—	0	0	*0*	*0*	*0*
75	A	4	15	—	22	26	55	102	A	4	20	22	22	55	55	251*	A	4	1	3	—	51	91
		22	16	—	19	23	*32*			22	8	10	17	25	25			22	4	—	7	—	56
	C	—	2	—	0	2	0		C	—	2	1	1	2	1		C	—	3	—	1	1	3
	G	—	0	—	0	0	0		G	—	3	3	3	3	3		G	—	0	0	—	0	0
8	A	4	—	18	55	55	45	19	A	4	24	—	14	50	28	321	A	4	55	56	67	67	55
14*	A	4	6	4	—	2	20	37	A	4	14	17	—	20	*20*			22	30	36	55	45	30
25	A	4	61	61	50	61	—	85	A	4	29	34	41	35	*41*		C	—	4	4	2	2	1
26	A	4	2	—	2	5	7	96	A	4	34	42	42	50	61		G	—	0	0	0	0	0
27	A	4	48	42	50	50	61	130	A	4	—	8	17	100	*45*	23*	A	4	29	42	—	42	*42*
31	A	4	50	56	50	48	50	132	A	4	25	38	75	—	*58*	61	A	4	29	—	61	—	*7*
38	A	4	50	34	50	—	60	147	A	4	82	82	82	82	*82*	65*	A	4	—	*15*	*91*	—	*110*
47	A	4	—	15	12	24	42	153	A	4	29	29	56	—	*50*	120*	A	4	74	—	74	91	—
49*	A	4	61	61	61	61	—	156	A	4	1	1	1	2	*9*	173	A	4	61	—	61	74	91
54*	A	4	—	31	26	26	18	242	A	4	—	13	13	17	*3*	175*	A	4	17	—	24	—	*24*
56*	A	4	46	38	—	46	—	311	A	4	7	17	67	—	17	217*	A	4	5	8	8	*5*	*5*
63*	A	4	7	—	24	24	23	341	A	4	2	34	7	6	*5*	234*	A	4	9	9	—	*183*	*146*
77	A	4	1	2	7	—	31	345	A	4	23	28	34	50	*41*	378	A	4	46	81	100	*133*	—
81	A	4	*42*	—	91	—	61																
262	A	4	9	—	9	—	61																
273	A	4	16	—	34	50	50																
286*	A	4	29	—	34	50	50																
292*	A	4	12	31	—	—	31																
Mean	A	4	25.5	31.5	36.1	37.3	40.0	Mean	A	4	22.5	28	34.4	41.3	36.6	Mean	A	4	34.3	38.6	59.1	76.6	66.9

In 10 of the cases titers of inhibitor against the hyaluronidase of a strain of *Strep. hemolyticus* of group A, type 22, and strains from groups C and G are included in addition to the inhibitory titers against the enzyme produced by the type 4 strain.

Titers in italics are those done on serum specimens obtained during administration of salicylate.

* Cases indicated by an asterisk are those in whom antistreptokinase and antistreptolysin 0 titers have been reported by Anderson, Kunkel and McCarty.[12]

week period after the onset. In cumulative figures, 3 reached a significant rise before the 15th day, 24 before the 29th day, and 9 after the 28th day.

3. Comparison of changes in hyaluronidase inhibitory titer with the changes in serological tests specific for the hemolytic streptococcus.—Of the patients included in this study, 16 were also recently reported on by Anderson, Kunkel and McCarty. In their work determinations of antistreptolysin 0 and antistreptokinase titers were done on the weekly bleedings. It is possible to compare, therefore, the changes in the three tests in these 16 patients. The data are found in their report,[12] and the cases included in their study are indicated by an asterisk in table 3. Of these 16 cases, 12 showed a significant rise with the antistreptokinase test and 10 with the antistreptolysin 0 test. An increase in hyaluronidase inhibition titer of 70% or more occurred in 9 of the cases. Seven of the 9 had an increase in titer in both of the other tests and two in neither. The relative height and amount of change in titer of hyaluronidase inhibitor was usually about that found by the other tests.

4. Comparison of the inhibitory titers in the rheumatic and non-rheumatic groups.—There was no appreciable difference between groups 1 and 3 in the number of cases showing a significant increase in titer. The mean weekly titers of the cases are shown in table 3. Because of the wide range of the values and overlap in the two groups, the difference between these means is not significant. There is less overlap when the maximum titers reached in each case in the two groups is compared, but the difference here is not highly significant either, there being 1 chance in 20 that such a difference would occur by chance alone. Although these are not significant differences, they support our previous impression that rheumatics tend to have higher levels of hyaluronidase inhibitor than do others following streptococcal infections.

Seven of the 13 cases of rheumatic fever (group 3) had an abrupt large rise in titer, and in these cases the onset of rheumatic symptoms usually started during or slightly before this rise.

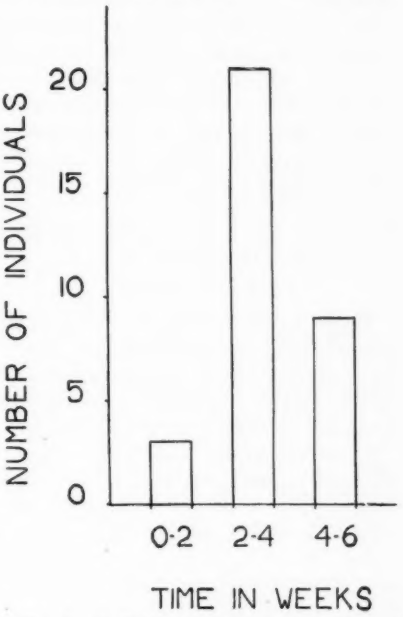

FIG. 3.—Individuals having a rise in titer of 70% or more arranged according to the time when this point was reached in two week periods after the onset of illness.

However, the relationship of the rise in titer to the onset of the scarlet fever in these 7 cases was such that most antibodies would also be expected to increase at this time. If the titer changes are compared with those reported for the antistreptokinase and antistreptolysin 0 titers by Anderson, Kunkel and McCarty, this can be seen to be the case. Three of the cases of rheumatic fever showed no change in titer of hyaluronidase inhibitor, even though the antistreptolysin 0 or antistrep-

tokinase did show a significant change. These three cases were infected with a type 17 strain of streptococcus. If the apparent failure of the type 17 streptococci studied here to stimulate production of hyaluronidase inhibitor were borne out by further studies, these three cases would be a strong argument against any possible relationship of streptococcal hyaluronidase to the pathogenesis of rheumatic fever. With the possible exception of these three cases, the changes in hyaluronidase inhibitor seemed to conform to those observed in the accepted streptococcal antibody tests during rheumatic fever.

The range of action of the inhibitor against hyaluronidase from various sources

Other workers, using different methods of testing for hyaluronidase activity, have reported that normal animal and human serums contain inhibitors of testicular hyaluronidase. Using the mucin clot prevention test, however, our normal animal serums did not cause inhibition of testicular hyaluronidase.[1] Tests were made to determine whether or not human serums contained such inhibitors. It was also decided to determine if the range of action of the inhibitor under study was similar to that of antiserums prepared in rabbits against group A streptococcal hyaluronidase.[14]

Thirty serums with high titers of inhibitor of the group A, type 4 streptococcal hyaluronidase were tested against the enzyme in an extract of bull testicle.* The serums were tested at both 1:10 and 1:200 dilutions. No inhibitory effect was observed.

Serums from 10 of the cases of scarlet fever were tested against the hyaluronidase from streptococci of group A, type 22, group C, and group G, in addition to being tested against that produced by the group A, type 4 organism. The results of these tests are included in table 3. It will be seen that the titer of inhibitor against the enzymes of the two types of group A streptococci showed a fairly close parallel, although they were not usually identical. By contrast, there was very little inhibition of the group C or G enzymes. There was no consistent difference, in the range of action of the inhibitor against hyaluronidase from the different sources tested, between cases that developed rheumatic fever and those that did not. The high dilution at which these serums were tested might have obscured such a difference if it had been present. It may be said that the inhibitor of streptococcal hyaluronidase in these serums was highly specific for the enzyme of group A organisms, and was not appreciably different in its effect against hyaluronidase from type 4 or type 22.

The inhibitory effect of pooled purified human γ globulin

A solution of pooled purified human γ globulin was tested for the presence of an inhibitor of the type present in whole human serum. The preparation used was about a 16% solution.* A 1:200 dilution of this preparation, which contained about 20 times the concentration of γ globulin as the same dilution of serum, caused complete inhibition of the undiluted enzyme in the test. A 1:400 dilution had an inhibitory

14. McClean, D. 1942, Methods of assay of hyaluronidase and their correlation with skin diffusing activity. Biochem. J. **37**: 169–177.

* These serums were also tested against the enzyme of the same strain of *Staph. aureus* used previously and against hyaluronidase from streptococci from groups B, C, and G. The results of the tests conform with those reported earlier[1] and here, and are therefore not given in detail.

* Immune serum globulin, human, E. R. Squibb and Sons. According to Dr. John W. Palmer of the Squibb protein fractionation laboratories, electrophoretic analysis of the lot used here showed it to contain 96% γ globulin, 2% β globulin and 2% albumin.

titer of 158. Making an allowance for the added dilution, the inhibitory effect was concentrated about 12 times in the γ globulin solution, when the mean titer of 27 of the initial bleedings in the 50 cases is used for comparison. Tests on similar preparations of γ globulin have revealed various antibodies to be concentrated from 10 to 34 times.[15] The inhibitory titer against group C hyaluronidase of this same dilution of γ globulin was 6. This specificity of effect of the inhibitor is similar to that observed for the inhibitory effect of whole serum.

The inhibitory titer of the γ globulin solution being tested was consistently lower after heating at 56 C for 30 minutes. The decrease in titer during this period was 20 to 40%. Heating of γ globulin at 56 C for 15 minutes had no consistent effect on the inhibitory titer. These results are shown in table 2. The effect of heating at 56 C on the inhibitory effect of γ globulin was not identical with the effect of similar heating on the inhibitor of streptococcal hyaluronidase contained in whole serum. Heating at 56 C for 15 minutes had no effect on the titer of either of these, however, while the inhibitor described by Haas[4] was reported to be completely destroyed by a similar period of exposure to this temperature.

DISCUSSION

Reports of the presence of hyaluronidase inhibitors in serum have appeared in the past and have recently aroused renewed interest. Inhibition of the ability of hyaluronidase to reduce the viscosity of hyaluronic acid solutions,[4,5] to decapsulate streptococci,[8] to disperse the follicle cells surrounding the ovum,[6] and to speed the spread of dye through the subcutaneous tissues[7] have been the test methods used. The reports do not clarify the nature of these inhibitory substances, but there is agreement that they are active against hyaluronidase from testicular extracts and that they are present in most animal serums tested. It is claimed that these inhibitors are rapidly destroyed by heating for 15 minutes at 56 C.[4]

It has not been possible to demonstrate such an inhibitor of testicular hyaluronidase in normal animal serums with the mucin clot prevention test, nor has it been possible to show that human serums with high titers of inhibitor of streptococcal hyaluronidase had demonstrable inhibitory titers against the enzyme in testicular extracts. It has been shown instead that the inhibitor under study has little or no effect even against the hyaluronidases of streptococci closely related to group A.

These two types of inhibitors may also be contrasted in regard to their degree of resistance to heating at 56 C. A significant loss in titer of inhibitor of streptococcal hyaluronidase occurs only when the serum is heated at 56 C for more than two hours. By contrast, Haas[4] has reported that 84% of the inhibitor studied by him is destroyed by heating at 56 C for 15 minutes. The serums tested in this study were all heated for 30 minutes at 56 C before being tested, and it does not seem likely, therefore, that inhibitors of the heat labile type contributed to the titers obtained in the tests.

The confusion between these groups of hyaluronidase inhibitors is lessened if one recognizes that they are determined by different methods of hyaluronidase assay. These methods are apparently concerned with different stages of hyaluronic acid breakdown,[16] and so the

15. Enders, J. 1944. Chemical, clinical, and immunological studies on the products of human plasma fractionation. X. The concentration of certain antibodies in globulin fractions derived from human blood plasma. J. Clin. Investigation **23**: 510–530.

16. Meyer, K. 1947, The biological significance

inhibitors would seem to be acting on different steps in the reaction.

The inhibitory effect of salicylate on hyaluronidase was first demonstrated by Guerra,[17] when he reported that administration of salicylate inhibited the rapid spread of dye which occurs when a mixture of dye and hyaluronidase are injected intracutaneously. Other observers soon reported that salicylate also inhibited hyaluronidase in vitro, but only in concentrations of the drug greater than those reached clinically and great enough to cause denaturation of other proteins.[18,19] However, it has more recently been shown that gentisic acid, which is excreted in the urine during salicylate therapy, is a potent hyaluronidase inhibitor at rather low concentrations.[20,21] These reports suggest that potent hyaluronidase inhibitors may be present in the body fluids of patients receiving salicylates. It becomes important, therefore, to examine the data presented here rather closely in order to determine what effect such substances may have had on the results obtained.

If salicylate or its derivatives acted as a hyaluronidase inhibitor in these serums, it would be natural to expect some upward change in the inhibitory titer in many or most of the cases at the start of salicylate administration.* Obviously such a factor is not easy to evalutate in the presence of titers already undergoing change. It was observed, however, that only 4 of the 22 cases that received salicylate showed a significant rise in titer following initiation of salicylate therapy. It was also observed that a significant change in titer occurred in the same percent of the cases that received salicylate as in those that did not. It happens, as can be seen in table 3, that in most of the cases that received salicylate, administration of the drug was started just prior to the last bleeding. A significant rise in titer occurred at this time in 20% of these cases. The fact that the same percent of cases that received no salicylate also showed a rise in titer at this time is a further point against any possible influence of salicylate or its derivatives on the inhibitory titers.

The data presented here give some indication as to the probable nature of the inhibitor under study. In the majority of the 50 cases of scarlet fever, the titer of this inhibitor in the serum rose to a greater degree than could be accounted for by the range of error of the test. In the cases showing a rise in titer the maximum point was usually reached more than two weeks after the onset of the disease. The correlation between the changes in titer of this inhibitor and those obtained in tests for antistreptolysin 0 and antistreptokinase was fairly close. The inhibitor was highly specific for the hyaluronidase produced by hemolytic streptococci of group A. Pooled purified human γ globulin contained an inhibitor with the same specific effect against group A streptococcal hyaluronidase. The inhibitory titer of serums from newborn infants were in

of hyraluronic acid and hyaluronidase. Physiol. Rev. **27**: 335–359.

17. Guerra, F. 1946, Action of sodium salicylate and sulfadiazine on hyaluronidase. J. Pharm. & Exper. Therap. **87**: 193–197.
18. Pike, R. 1947, Failure of sodium salicylate to inhibit hyaluronidase in vitro. Science **105**: 391.
19. Dorfman, A., Reimers, E. and Ott, M. 1947, Action of sodium salicylate on hyaluronidase. Proc. Soc. Exper. Biol. & Med. **64**: 357–360.
20. Meyer, K. 1948, Hyaluronic acid and the rheumatic diseases. Mod. Concepts of Cardiovasc. Dis. XVII, no. 2.
21. Meyer, K. and Ragan, C. 1948, Inhibition of hyaluronidase by hydroquinones and quinones. Federation Proc. **7**: 173.

* Serums obtained during periods of salicylate therapy are indicated in italics in table 3.

the same range as the titers of serum from adults. On the other hand, serums from young individuals over three months of age usually had very low titers. This suggests that the inhibitors in the serum of newborn infants is acquired from the mother and lost during the first few weeks of life. These characteristics all support the possibility that the inhibitor under study may be an antibody.

Streptococcal hyaluronidase has been shown to be antigenic, and antiserums prepared in rabbits have been shown to be group specific.[14] Studies of hyaluronidase production by group A streptococci using the mucin clot prevention test have shown production of the enzyme by only types 4 and 22.[22] None of the streptococci isolated from these cases were of types 4 or 22, and none could be shown to produce hyaluronidase when tested by the mucin clot prevention test. This finding may be cited as evidence against the possibility that the inhibitor is an antibody. It has already been suggested that an inactive form or precursor of hyaluronidase might be the stimulus for antibody production, or that enzyme might be produced in vivo but not in vitro.[1] Streptolysin O requires chemical treatment in the form of reduction before it is demonstrable in streptococcal culture filtrates, even though antibodies are regularly produced during streptococcal infections; and the situation with regard to hyaluronidase might be analogous to this. It is also possible that many strains may produce hyaluronidase in amounts too small to be demonstrable by the mucin clot prevention test. This possibility is supported by Pike,[23] who recently reported that most strains of group A streptococci produce amounts of hyaluronidase demonstrable by the more sensitive turbidometric method even when the mucin clot prevention test gives negative results.

SUMMARY

Further studies have been done on an inhibitor in human serums of the hyaluronidase derived from a strain of *Streptococcus hemolyticus* of group A, type 4. The titer of this inhibitor in the serum increased during convalescence in most of 50 cases of scarlet fever. The maximum titer of the inhibitor was reached more than 14 days after the onset of the infection in most of the cases. The inhibitor was highly specific for the hyaluronidase of the group A streptococcus, and the changes in titer approximately paralleled the changes in titer of antistreptokinase and antistreptolysin O. An inhibitor with a similar specific effect on the hyaluronidase produced by group A streptococci was shown to be concentrated in pooled purified human γ globulin. The inhibitor in whole serum was stable at 56 C for relatively long periods. The distribution of titers among serums from infants and children was such as to suggest that transfer of the inhibitor from the maternal to the fetal circulation occurs, and that the passively acquired inhibitor is lost during the post-natal period. Certain of these findings differentiate this inhibitor from the inhibitor of testicular hyaluronidase currently under study in other laboratories. The findings reported here indicate that the inhibitor under study here is probably of the nature of an antibody.

22. Crowley, N. 1944, Hyaluronidase production by hemolytic streptococci of human origin. J. Path. & Bact. **56**: 27–35.

23. Pike, Robert M. 1948, Streptococcal hyaluronic acid and hyaluronidase. I. Hyaluronidase activity of non-capsulated group A streptococci. II. Production and subsequent destruction of hyaluronic acid by certain strains of group A streptococci. J. Infect. Dis. **83**: 1–18.

BLOOD p_H AS A FACTOR IN GENETIC RESISTANCE TO MOUSE TYPHOID

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The problem of genetic resistance to mouse typhoid has been approached by differentiating, through selective breeding, strains of mice which show marked differences in resistance. At the Iowa State College 6 such strains have been developed, each breeding true to its particular level of resistance. In terms of survival from intraperitoneal inoculation with a standard dose of 200,000 organisms of *Salmonella typhimurium*, the 6 strains range from 86% survival to 8% survival. On the basis of considerable evidence it has been established that resistance to mouse typhoid is highly specific and that the inheritance is complex (Gowen and Schott, 1933a; Hetzer, 1937; Webster, 1937 and Hill et al., 1940).

Inbred strains of mice, each exhibiting a predictable response to artificial infection, afford unique material for a critical examination of the physiological basis of resistance. Once the chemical nature of a natural defense mechanism is understood, the application of preventive measures may be but a step, so that in the end even genetically susceptible individuals may be protected. Logically, the first step in such an undertaking is to discover what inherited characters in the host are responsible for resistance or susceptibility. This phase of the problem is under investigation in the genetics laboratory at the Iowa State College.

It is the purpose of this paper to present evidence on the differences between inbred strains of mice in blood p_H and the bearing such differences may have on the inherited level of resistance.

Since Loeffler (1892) first described mouse typhoid, it has been investigated from the standpoints of (1) descriptive histo-pathology, (2) epidemiology, (3) genetics of resistance and susceptibility and (4) the physical basis of genetic resistance. It is the last of these topics with which the present study is primarily concerned. The genetics of the host as a factor in resistance to mouse typhoid was mentioned in the early epidemiological studies (Topley, 1919 and Webster, 1923), but only in recent years have inbred strains, differentiated on a basis of genetic resistance to disease, been available for investigating the nature of genetic resistance. Reviews dealing with the inheritance of resistance to disease in animals include those of Gowen (1933 and 1948), Hill (1934), Webster (1937) and Perla and Marmorsten (1941).

A few instances are known in which host resistance to disease can be referred to specific genes. These include resistance to *Bacillus piliformis* in mice

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(Gowen and Schott, 1933b) and deficiency of complement in the blood of guinea pigs (Rich, 1923). Mouse typhoid, in that it is governed by multiple genetic factors for resistance and susceptibility (Hetzer, 1937), is more representative of animal diseases in general.

Resistance to different infective agents is not necessarily determined by the same genes. For example, Gowen and Schott (1933a) found that mouse typhoid resistant S and susceptible sil mice were quite different in their reaction to pseudorabies virus. On a basis of survival, sil mice proved superior to S in resistance to the virus.

Not only is the inheritance of resistance, as expressed in terms of per cent survival from artificial infection, a complex affair, but the mechanisms which contribute to resistance to a particular disease are also likely to be influenced by several or many genes. Before probing the problem more deeply at the chemical level it would appear profitable to correlate certain measurable attributes of inbred strains of animals with the resistance level. This has already been done for several characteristics. Irwin and Hughes (1931 and 1933) found that the bactericidal power of whole blood partially accounted for survivals in their rat strain which was resistant to *Salmonella enteritidis*. Hill et al. (1940) demonstrated that inheritance of resistance to the partially purified endotoxin from *S. typhimurium* is independent of resistance to live organisms. Gowen and Calhoun (1943) found a high correlation between total leucocyte number and resistance to mouse typhoid in normal mice of 6 inbred strains. Recently Oakberg (1946), using the same 6 mouse strains, found a correlation between resistance and the ability of Kupffer cells of the liver and reticular cells of the spleen to digest bacteria.

This paper presents a study of the genetics of blood p_H in the same strains. Variations in blood p_H with particular emphasis on human subjects have been reported by a number of investigators. Work prior to 1926 has been summarized by Austin and Cullen (1926) and the p_H limits reconsidered by Earle and Cullen (1929). Using the colorimetric method, they established the normal range to be p_H 7.30–7.52. On the basis of a later correction it became apparent that these values were on the average 0.07 p_H too high (Myers and Muntwyler, 1940). Schock and Hastings (1934) reinvestigated variations in p_H of the blood in humans and found that normal individuals differ significantly from one another. They also found a slightly higher p_H in women than in men.

Changes in blood p_H which accompany experimental infection have some bearing on the present study. Dragstedt (1920) demonstrated a decline of blood alkalinity in rabbits infected with *Streptococcus hemolyticus*. He found no evidence to support a then current theory that acidosis is in itself a mechanism of resistance. The p_H of the blood varied from a normal of 7.65 to 7.3 in an extreme case. Other studies (Hirsch, 1921 and Hirsch and Williams, 1922) showed a lowering of the alkalinity and alkaline reserve of the blood in the experimental infection induced by each of 5 different pathogens. Rabbits in which the p_H was lowered moderately recovered; those with a marked lowering died. In two cases the hydrogen ion concentration became so great that the reaction became slightly acid.

Changes in the alkali reserve of blood with parallel observations on the variation in number of leucocytes were studied by Hirsch (1921). Intravenous injection of living bacteria into rabbits resulted within 2 to 4 hours in a fall in leucocyte number, in some cases to as

low as 1,000 to 2,000 per cmm, and at the same time in a diminution of the blood alkali reserve. There followed a rise in the number of leucocytes and a return to normal of the alkali reserve or even a short period of alkalosis. The author suggested that the fall in alkali reserve or factors associated with it might provide the chemical stimulus for leucocytosis. Gräff (1922) referred the migration of leucocytes to the navel at the time of labor to a lowering of the p_H in the amniotic fluid.

Although variations in blood p_H are normally restricted to a relatively narrow range, detection of small shifts in p_H may indicate much larger ones in local areas somewhat removed from the main blood stream. Menkin (1934) demonstrated that replacement of polymorphonuclear cells by macrophages in inflammatory pleural exudate depends on the production of a local acidosis. That the p_H exerts a primary and direct effect on the cellular sequence in an inflamed area is further supported by the studies of Evans (1931) and Menkin (1939). Whereas leucocytes are injured when exposed to a p_H of 6.6, macrophages are apparently undamaged. The p_H and differential counts of the blood, however, remained relatively stable when pleural exudation was induced by injecting turpentine into the chest. (See data of Menkin, 1934, and Menkin and Warner, 1937.)

Several events formerly attributed directly to depletion in alkaline reserve and change in p_H are now referred to the action of specific factors. Thus "leukotaxine" is concerned with local migration and diapedesis of polymorphonuclear leucocytes (Menkin, 1938), a "leucocytosis promoting factor" induces a rise in the number of circulating leucocytes (Menkin, 1940a and b), and "necrosin" accounts for leucopenia (Menkin, 1943 and 1944).

From the literature there is no good evidence that blood p_H is a primary mechanism of resistance, but its relation to other known defense reactions suggests that it may be an index of resistance. With that viewpoint, and using resistant and susceptible inbred strains of mice, the present investigation was undertaken.

MATERIALS AND METHODS

Five of the 6 highly inbred strains of mice described by Gowen and Calhoun (1943) and Oakberg (1946) have been used in this study.* Two strains, RI and S, are highly resistant, two strains, L and Ba, are susceptible and the Z strain is intermediate in resistance to mouse typhoid. These distinct strains, which have been maintained by full sib matings for upwards of 20 generations, were formed by different breeding techniques. The RI and S strains were produced by inbreeding with selection for resistance, the L strain by inbreeding with selection for susceptibility and the Ba and Z strains by inbreeding without selection for either resistance or susceptibility.

Adult mice were used almost exclusively for blood p_H determinations since there was found to be no age effect on p_H among mature animals. Mortality following bleeding was very much lower in the older, larger mice. Blood samples were obtained by cardiac puncture of non-anesthetized mice using a 25 or 26 B-D needle and 0.5 cc tuberculin syringe. No anticoagulants were added to the blood but the needle and syringe were rinsed with a 4% sodium citrate solution between samples. The p_H readings were made at room temperature.

Since Kerridge (1925) called attention to its use for biological fluids, the glass electrode has come into use for measurement of the p_H of whole blood. The present investigation was carried out with a research type Cambridge electron-ray p_H meter equipped with a micro glass electrode of 0.4 cc capacity. The instrument was balanced before each experiment, using standard buffers of p_H 4.00 and 7.00; and in addition, a series of buffer solutions of known p_H were tested at intervals to check the accuracy of the instrument.

* The names attached to these particular strains only indicate the source of the original stock. They are not to be confused with other strains passing by these names, for while they stem from the originals in each case, they differ from other like named strains in many particulars.

Infection with mouse typhoid was accomplished by intraperitoneal inoculation of live organisms. The 11 c strain of bacteria described by Zelle (1942) was used. Organisms grown on nutrient agar and incubated for 18 hours at 37 C were suspended in peptonized physiological saline. The number of organisms was approximated by means of a Gates Nephelometer and appropriate dilutions made. To immunize mice, suspensions were prepared by heating at 60 C for 30 or more hours until the organisms were killed.

EXPERIMENTAL RESULTS

Blood p_H of inbred mouse strains

The 5 strains of mice fall into three populations with respect to blood p_H. Combined data from table 1 show the S and RI resistant strains have a mean p_H of 7.430, L and Ba susceptible strains a p_H of 7.359 and the Z strain of intermediate resistance a p_H of 7.285. The sex classification has been omitted because there was no significant difference between sexes in any of the strains or their crosses. The mean difference between 342 males and 282 females was .007 p_H.

TABLE 1.—*Strain differences in blood p_H and comparative resistance to mouse typhoid.*

Strain	p_H of reduced blood		Resistance*	
	Number tested	Mean p_H	Number tested	% survived
S	200	7.427	1150	86
RI	116	7.434	496	75
L	96	7.355	470	13
Ba	236	7.361	452	8
Z	141	7.285	1262	58

* Gowen & Calhoun, 1943. Dose: 200,000 organisms 11c culture *S. typhimurium*.

Concomitant with the fixation of characteristic levels of resistance to mouse typhoid, the inbreeding has fixed different blood p_H levels. Since the parental stocks from which the strains were derived differed widely in origin, and the breeding techniques by which the different strains were formed also varied, the correspondence between resistance levels and blood p_H is suggestive. Were the two resistant and two susceptible strains the only ones available for study, there would have been a seemingly much stronger case for the association of relatively high blood alkalinity and resistance. The fact that the Z mice, possessing a moderate level of resistance, have the highest hydrogen ion concentration (lowest p_H) makes it clear at the outset that low p_H is not necessarily inimical to resistance.

TABLE 2.—*Strain differences in blood p_H in two experiments.*

Mouse strain	Experiment I		Experiment II	
	Number of mice	p_H	Number of mice	p_H
		Mean S.E.		Mean S.E.
S	14	7.431 ± .018	30	7.410 ± .010
RI	14	7.434 ± .018	30	7.398 ± .013
L	14	7.363 ± .018	30	7.329 ± .012
Ba	14	7.372 ± .020	30	7.344 ± .009
Z	14	7.300 ± .019	30	7.273 ± .014

Appreciation that p_H may vary from one day to the next led to adoption of experimental designs that would insure the validity of comparisons between strains or treatments. Unequal subclass numbers were obviated by having extra mice on hand for each experiment. Samples of blood which were unsatisfactory for p_H testing due to the presence of bubbles or insufficient quantity were rejected without testing. Small fluctuations in the p_H meter may occur during an experiment due to liquid junction potentials and thermal and electrical hysteresis. For this reason the order in which mice were tested within each set of observations was arranged according to a latin square.

Data from two such experiments showing means and standard errors are summarized in table 2. These data are combined with those of other experiments involving pure strains to make up table 1. In the experiment designated I in table 2, two mice from each strain were tested for p_H on a given day. This procedure was repeated every other day

until 7 such groups had been tested. The second experiment, II, differed in that 5 mice from each strain were tested on a given day, the interval between tests was one week and the tests covered a period of 5 weeks.

There was a significant difference between experiments I and II, but differences between means for the various strains were of the same order. Combining S and RI strains to obtain means of p_H 7.433 for experiment I and p_H 7.404 for experiment II and combining L and Ba to get corresponding values of 7.368 and 7.337, the differences in means between the resistant S and RI and susceptible L and Ba are 0.065 p_H and 0.067 p_H. Similarly the means for L and Ba exceed the Z means by 0.068 and 0.064 p_H. The differences between means from all data for pure strains are: S and RI minus L and Ba, 0.071; L and Ba minus Z, 0.074. The three p_H levels found in the 5 mouse strains are separated by differences of 0.064 to 0.074 with a maximum difference between means of 0.161 p_H.

Considering experiments I and II of table 2 separately, the variation in blood p_H may be divided into that which occurs between mouse strains and that which occurs between days of test and the interaction. The error variance represented by mice treated alike represents errors in measurement as well as variations in p_H between mice within strains. Results of the variance analysis are shown in table 3. The differences between means of strains (table 3) indicate that genetic differences between mice in blood p_H have been stratified at different levels in the various inbred mouse strains.

TABLE 3.—*Analysis of variance for the effects of strain and day of test on blood p_H.*

Experiment	Source of variation	d/f	Mean square	F	Components of variance
I	Mouse strain	4	0.0427	11.24†	E+2I+14G
	Day of test	6	0.0148	3.89†	E+2I+10D
	Interaction	24	0.0038	1.03	E+2I
	Mice treated alike	35	0.0037		E
II	Mouse strain	4	0.0920	17.69†	E+5I+30G
	Day of test	5	0.0101	1.94	E+5I+25D
	Interaction	20	0.0052	1.53	E+5I
	Mice treated alike	120	0.0034		E

† Significant beyond the 0.01 level.

An analysis of the relative contributions of genetic and environmental factors is summarized in table 4.

The variance analyses gave essentially the same picture for the two experiments. Roughly 50% of the variation in blood p_H is due to unknown causes of variation and variation within mice, 40% is due to genetic differences between strains and 10% to variation between days of test. The strain differences of 40% may be contrasted to figures of 22% for erythrocytes and 26% for leucocytes reported by Gowen and Calhoun (1943).

Frequency distributions showing variations in blood p_H of the strains are shown in figure 1. The data from experiments I and II, between which there is a significant difference, have been combined. Since the two experiments were conducted under somewhat different conditions and at different seasons of the year, the ranges should include those to be expected if a number of ex-

TABLE 4.—*Per cent contribution to total variance of blood p_H of strains, days of test and individual mice.*

Source of variation		Experiment I Percent	Experiment II Percent
Mouse strain	G	36	42
Day of test	D	15	3
Interaction	I	0	5
Mice treated alike	E	49	50

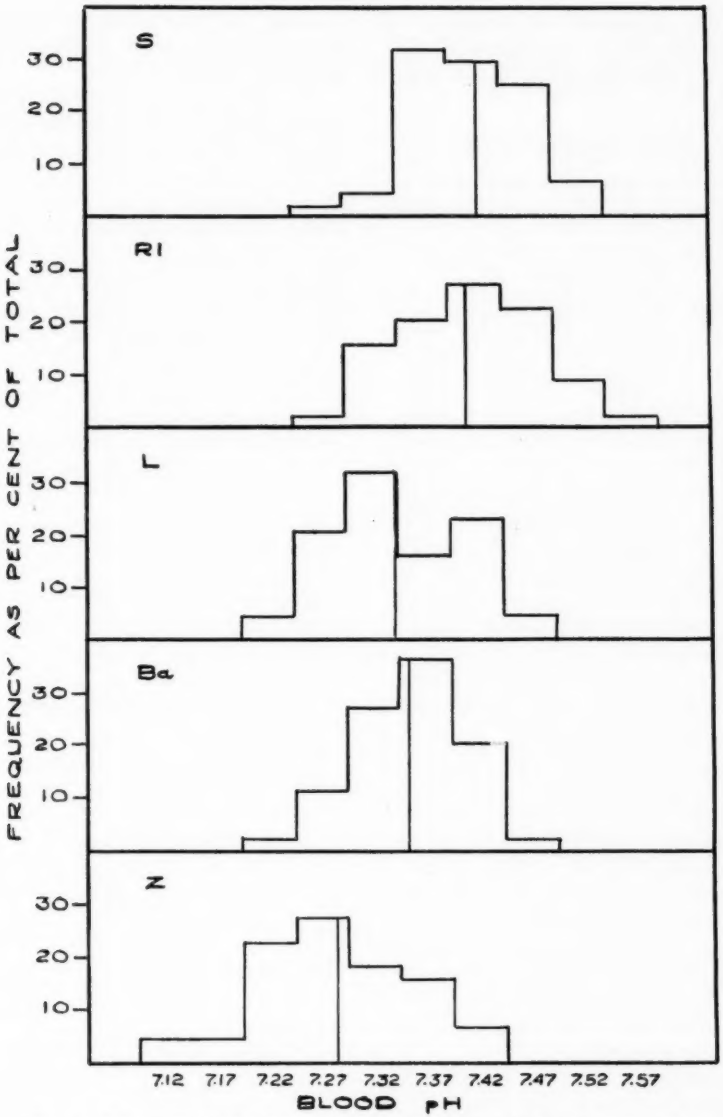

FIG. 1.—Frequency distributions and means of blood p_H of 5 inbred mouse strains.

periments were conducted under a fairly wide variety of conditions. Individual mice including all strains fell within 0.46 p_H. The ranges for the various strains were as follows: resistant strains (S and RI), 7.27–7.57; susceptible strains (L and Ba), 7.20–7.49; and the Z strain, 7.11–7.40. These figures

probably represent the extremes of normal variation. A narrower range must be expected in any single experiment.

Factors affecting blood p_H

Not only does the separation of distinct and genetically homogeneous lines of mice permit a high degree of genetic control, but, under laboratory conditions, environment may also be subjected to experimental control. The stability of blood p_H in an inbred line, for example, can be tested by contrasting the reaction of animals treated in various ways with untreated controls. Preliminary to any study of the effects of experimental treatments, it is of some interest to ascertain the nature and magnitude of those factors which most affect the reactions of untreated or control animals. There is always the possibility that such an analysis will permit differentiation between real and spurious correlations as well as lead to improvements in technique and in the design of future experiments. Experimental evidence has been obtained relevant to the effects of temperature, oxygenation, age and size.

TABLE 5.—*Blood p_H of mouse strains at 27 C ± 2 and at 24 C ± 1.*

Mouse strain	Experiment I p_H at 27 C ± 2	Experiment II p_H at 24 C ± 1	Difference
S	7.431	7.410	0.021
RI	7.434	7.398	0.036
L	7.363	7.329	0.034
Ba	7.372	7.344	0.028
Z	7.300	7.273	0.027
All strains	7.380	7.351	0.029

Temperature.—Most of the p_H measurements were made during the fall and winter months at a room temperature of 24 C ± 1. Freshly drawn blood samples were tested immediately and were not corrected to p_H values at 38 C. The data of table 2 are presented again in a slightly different form in table 5, showing the relationship between p_H and room temperature at the time the tests were made.

The difference of 0.029 p_H between determinations at 27 C and 24 C might be due to either the effect of temperature on the blood p_H in vitro or to temperature effects on the mice themselves. The first possibility would appear to be ruled out on a basis of experimental evidence that blood p_H in vitro falls with a rise in surrounding temperatures (Rosenthal, 1948). On this basis, blood samples tested at 24 C should have had the higher p_H. Evidently the differences between means for the two experiments cannot be accounted for by a temperature effect on the blood in vitro. To test the second possible interpretation of the experimental results, namely that the mice differed in blood p_H at the two temperatures, sib pairs of mice were placed in different rooms for 24 hours, one set at 32 C, the other at 24 C. The p_H determinations were all made at 32 C, and sib pairs were alternated. The results are shown in table 6.

Determined at 32 C, all the samples had a low p_H compared to those measured previously at 24 or 27 C (table 6). This is to be expected if p_H in vitro falls linearly with a rise in temperature. The differences between mice from the 32 and 24 C rooms, on the other hand, although not statistically significant, favor a hypothesis that the p_H of the blood in vivo goes up with a rise in sur-

TABLE 6.—*Blood p_H of sib pairs of mice held at 32 C and 24 C and tested at 32 C.*

Mouse strain	Number of sib pairs	p_H at 32 C: Mice from 32 C room	p_H at 32 C: Mice from 24 C room	p_H difference
RI	5	7.336	7.302	0.034
Ba	6	7.270	7.228	0.042*

* $P < 0.10$.

rounding temperature. Exercise may be the real answer, as it was observed that the mice in the cooler surroundings were much more active.

At this point it might well be argued that the whole question of strain differences in blood p_H merely resolves itself into a matter of body temperature relations in mice. An examination of the evidence shows that this is not the case. In the first place, RI mice with a relatively high blood p_H have a significantly lower body temperature than the other strains (Weir, 1947). Secondly, rectal temperature is apparently not correlated with blood p_H in any of the strains as shown in table 7. In this experiment two or three mice from each strain were tested each day for temperature and p_H, and the room temperature at which the mice were kept varied from 20.5 C to 28.5 C. Evidently variations in blood p_H are independent of body temperature, since none of the correlations are statistically significant.

All of the remaining experiments were conducted at 24 C. The p_H values as ordinarily determined are probably 0.03 to 0.05 p_H lower than those existing in the circulating blood (Myers and Muntwyler, 1940).

TABLE 7.—*Correlation of rectal temperature and blood p_H in 5 strains of mice.*

Mouse strain	Number of mice	Rectal temperature	p_H	Correlation
S	22	37.82 C	7.438	+0.151
RI	19	37.46 C	7.440	+0.346
L	18	37.86 C	7.363	+0.117
Ba	20	37.62 C	7.349	−0.248
Z	19	37.74 C	7.283	−0.167

Arterial versus venous blood.—Throughout the present study reduced blood was used as it was most easily obtained. Occasionally a sample of a much lighter hue, presumably from the left side of the heart, was obtained. Such samples were first recorded as "light red," then p_H tested and recorded separately. Over a period of time a number of sib pair observations were collected, one member with a record of the p_H of reduced blood, the other, oxygenated blood. Results are shown in table 8. The p_H difference of 0.115 between arterial and venous blood agrees with the figure of 0.114 for guinea pigs from the data of Hawkins (1924). Since the difference is the same for all strains it would not matter in strain comparisons whether arterial or venous blood were used so long as only one were chosen. Reduced blood was used throughout the present study.

TABLE 8.—*p_H of arterial and reduced blood from sib pairs of pure strains and crosses of mice.*

Mouse strain	Number of sib pairs	Blood p_H		p_H difference
		Arterial	Venous	
S	6	7.538	7.417	0.121†
RI	8	7.540	7.439	0.101†
Ba	7	7.470	7.358	0.112†
Z	1	7.40	7.30	0.10
F_1 S×Z	8	7.477	7.345	0.132†
F_1 Ba×Z	1	7.47	7.37	0.10
F_1 S×Ba	1	7.58	7.45	0.13
All strains and crosses	32	7.503	7.388	0.115†

† Significant beyond the 0.01 level.

Additional factors.—Single experiments designed to test the effect of age on p_H revealed a slightly lower p_H in immature mice in 2 of 4 strains tested. Consistent differences were found in Z and Ba mice and no differences in RI and S mice. The differences in means (0.01 to 0.03) were not significant.

Mature animals were used exclusively in all strains except the Z, in which some young animals were used.

Body size in the strains, determined for equal numbers of males and females at 90 days of age, are as follows: RI, 29 g; Ba, 23 g; S, 22 g; L, 21 g; and Z, 20 g. Evidently size per se has no bearing on either blood p_H or resistance. It was frequently observed that whenever a mouse became excited during handling

prior to cardiac puncture (generally the last mouse in the cage), a low p_H was recorded. This may have been due to the effect of adrenalin.

Blood p_H of F_1 and F_2 crosses

Progeny of a cross between the like p_H resistant strains S and RI did not deviate from the parental strains in blood p_H. Similarly the F_1 of the susceptible by susceptible, L×Ba, resembled their parents. Since there is no complementary effect of genes for blood p_H in resistant or susceptible crosses, one strain from each category was chosen for further crossing. These were the resistant S, susceptible Ba and intermediate Z strains. The S and Ba strains were chosen because they possess a higher level of fertility than the RI or L strain and represent the extremes in typhoid resistance.

TABLE 9.—*Blood p_H of S and Ba strains of mice contrasted with F_1 and F_2 crosses.*

		Number of mice	Blood p_H	
			Mean	S.E.
P_1	S	34	7.416	±0.008
	Ba	32	7.339	±0.010
Midparent F_1(S×Ba)		26	7.378 7.367	± .008
F_1-Midparent		difference =	−0.011	
F_2(S×Ba)		53	7.372	± .009

TABLE 10.—*Blood p_H of S and Z strains of mice contrasted with F_1 and F_2 crosses.*

		Number of mice	Blood p_H	
			Mean	S.E.
P_1	S	34	7.416	±0.008
	Z	41	7.288	±0.012
Midparent F_1(S×Z)		31	7.352 7.385	±0.014
F_1-Midparent		difference =	+0.033*	
F_2(S×Z)		53	7.345	±0.010

* Significant beyond the 0.05 level.

TABLE 11.—*Blood p_H of Ba and Z strains of mice contrasted with F_1 and F_2 crosses.*

		Number of mice	Blood p_H	
			Mean	S.E.
P_1	Ba	32	7.339	±0.010
	Z	41	7.288	±0.012
Midparent F_1(Ba×Z)		27	7.313 7.350	±0.016
F_1-Midparent		difference =	+0.037*	
F_2(Ba×Z)		53	7.316	±0.010

* Significant beyond the 0.05 level.

The P_1 crosses involving S, Ba and Z were made up by choosing one litter from each strain, each composed of 3 males and 6 females, and mating in all possible combinations of one male to two females. Progeny of the pure strain matings served as controls and a number of them were tested on every occasion that F_1 or F_2 mice were used. Since parental strain p_H values (tables 9, 10 and 11) agree rather closely with those reported in tables 1 and 2, it is apparent that the conditions under which the F_1 and F_2 tests were made were comparable.

Resistant by susceptible crosses.—Means and standard errors for the p_H measurement of S and Ba strains and crosses are shown in table 9. The F_1 mean falls midway between the two parents, indicating a net additive effect on phenotype of genes which differentiate the parental strains with respect to blood p_H. It also appears (fig. 2) that the inbred strains with ranges of p_H 7.32 to 7.52 for S and p_H 7.24 to 7.45 for Ba are more susceptible to environmental influences than the F_1 with a range of 7.31 to 7.44. Ancillary information came later from experiments directed toward altering blood p_H in which the S × Ba F_1 cross again proved to possess a more stable p_H. The standard deviations are S, 0.049; Ba, 0.056; F_1, 0.038 and F_2, 0.068. Recovery of parental values in F_2 with 53 individuals suggests that the difference between S and Ba in blood p_H may be conditioned by relatively few genes.

Resistant by intermediate and susceptible by intermediate crosses.—Crosses in-

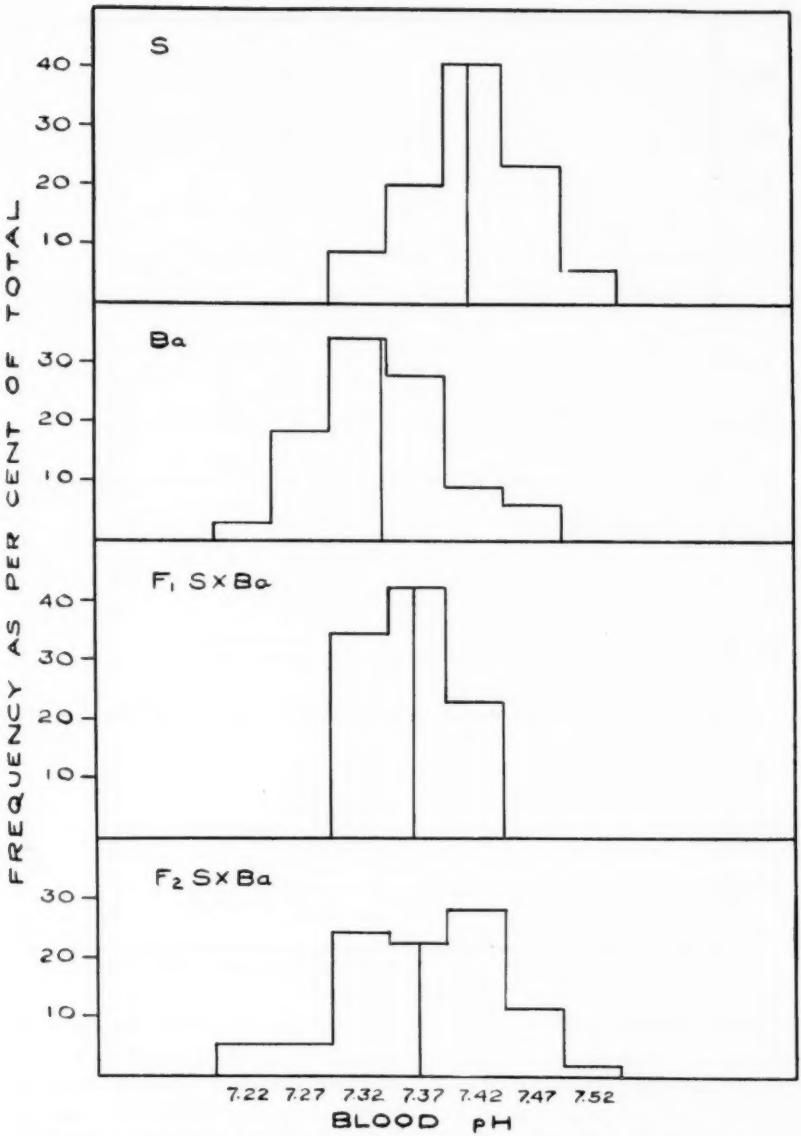

FIG. 2.—Frequency distributions and means of blood p_H of S, Ba, F_1 and F_2 crosses.

volving the Z strain present a somewhat different picture. The data are summarized in tables 10 and 11. Crosses of Z with either S or Ba produced F_1 progeny exceeding the midparent value. The difference of 0.033 p_H between S × Z mice and midparent and the difference of 0.037 p_H between Ba × Z and

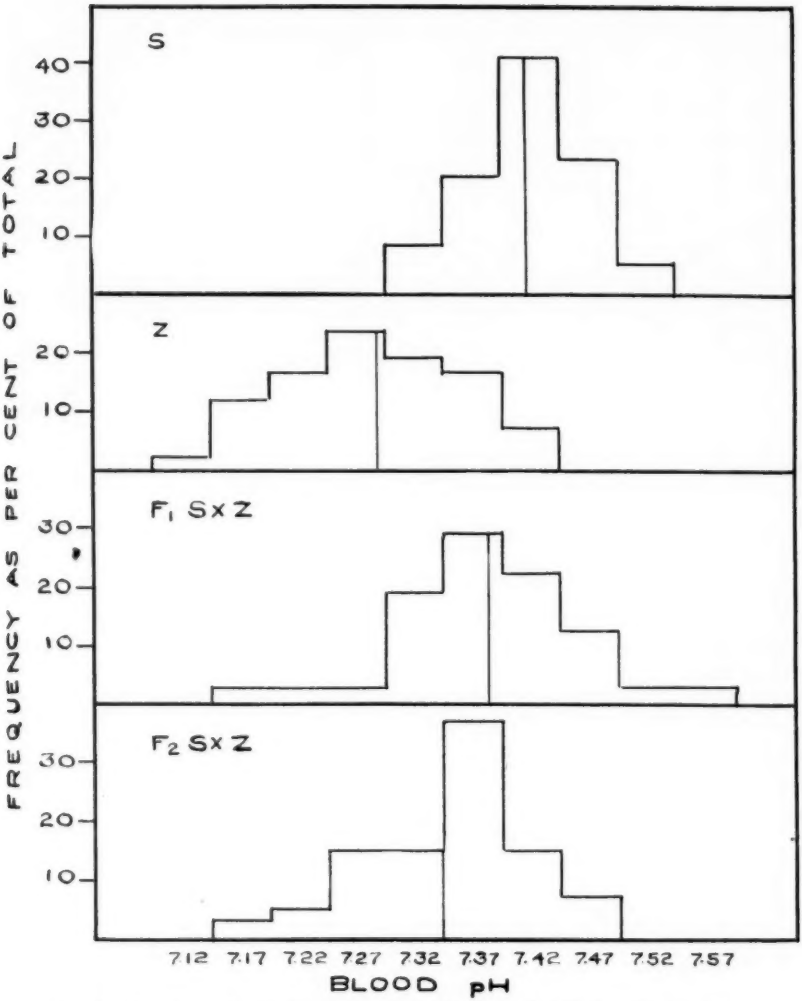

FIG. 3.—Frequency distributions and means of blood p_H of S, Z, F_1 and F_2 crosses.

midparent are statistically significant (tables 10 and 11). The F_2 p_H values correspond with the midparent.

In crosses with S or Ba the net phenotypic effect of genes responsible for low p_H in the Z strain is recessive. The Z crosses differ from the S×Ba crosses in other respects than dominance, as portrayed in the frequency distributions shown in figures 3 and 4. The F_1 ranges for S×Z and Ba×Z crosses exceed not only the parental strains but also the F_2 in each case. No completely adequate hypothesis can be advanced to account for the results. It appears that the Z strain possesses a heritable instability of p_H regulation. The standard deviations were Z, 0.078 p_H; S×Z F_1, 0.078 p_H;

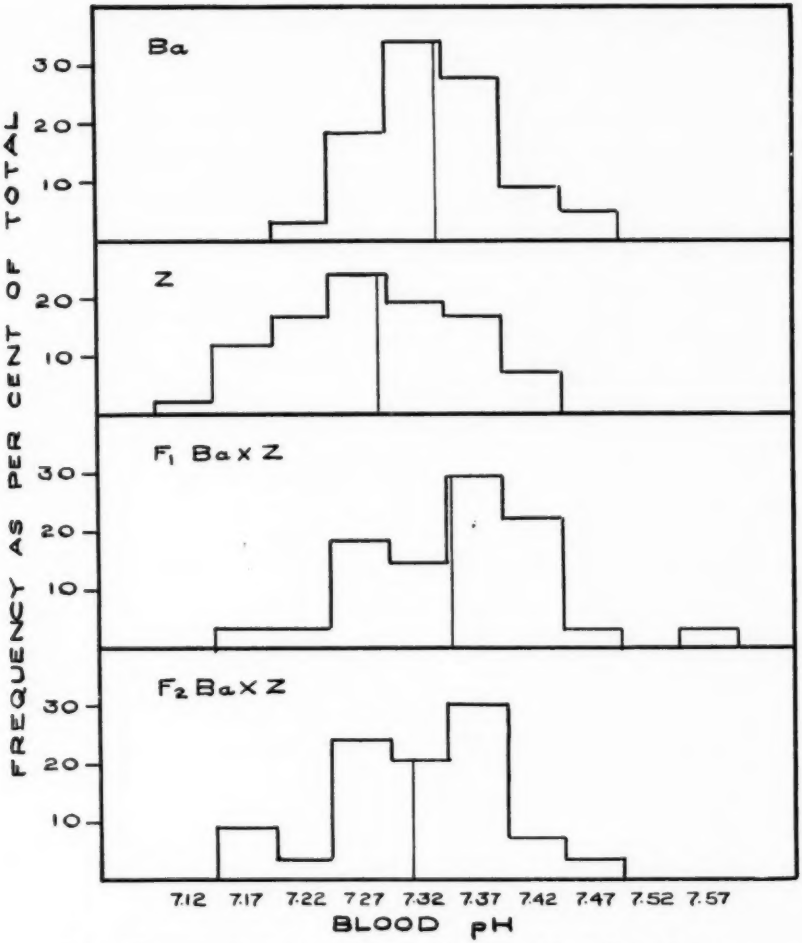

FIG. 4.—Frequency distributions and means of blood p_H of Ba, Z, F_1 and F_2 crosses.

F_2, 0.070 p_H; Ba×Z F_1, 0.082 p_H, F_2, 0.073 p_H. That decrease in genotypic variation due to inbreeding is not invariably accompanied by decrease in phenotypic variation is well illustrated by Wright's (1934) example in which the variation in number of digits in several inbred lines of guinea pigs was greater than that in the random bred control stock.

Changes in blood p_H with experimental infection

Mice showing marked clinical symptoms of mouse typhoid generally prove to have a lowered blood p_H. After the appearance of symptoms death follows so swiftly in susceptible strains that the time in which p_H changes may be followed is very short. Resistant mice, on

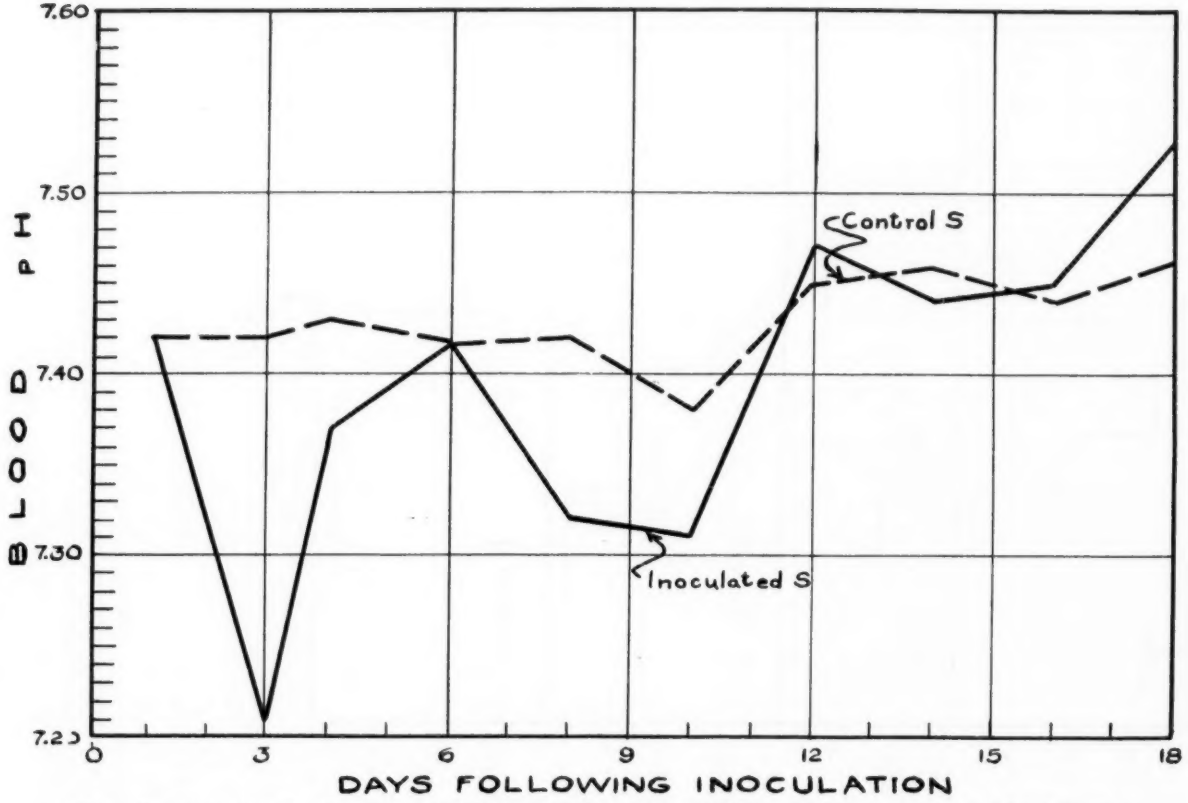

FIG. 5.—Changes in blood p_H with experimental infection. Inoculation dose: 500,000 live organisms of 11c strain of *S. typhimurium*.

the other hand, often recover from a severe manifestation of the disease and so offer favorable material to follow p_H changes in the course of an artificial infection.

Mice of the S strain were divided into two sib pair groups. One group was infected by intraperitoneal inoculation with 500,000 live organisms of the 11c strain of *S. typhimurium*; the other served as a control. Although both groups were kept in the laboratory isolation room after inoculation to insure uniform treatment, and hence the possibility of subclinical infection of controls could not be absolutely precluded, it is unlikely that chance infection did occur. Evidence for this comes from experience with Ba controls which have consistently survived even though an experimental dose of less than 100 organisms is sufficient to cause some deaths.

Results of an experiment with 27 sib pairs of S mice are shown in table 12 and figure 5. Of 7 mice which showed marked symptoms (ruffling of the hair, conjunctivitis and emaciation), 5 had a p_H well below their untreated litter mates. A number of infected mice not showing any marked effects of the disease also had a lowered p_H.

Since individual mice were bled and p_H tested only once, each point on the curves in figure 5 represents a mean p_H of three or sometimes two different mice. Changes in p_H over the 18-day period as represented in the inoculated group curve were influenced by sampling errors, since all affected mice did not become sick at the same time. It is of some interest that the period of lowered p_H corresponds with the time for which Seiffert et al. (1928) report a leucopenia.

Rectal temperatures were taken on 1, 6, 8, 10, 14, 16 and 18 days after inoculation. Mice with the disease had a

TABLE 12.—*Changes in blood p_H and rectal temperature with experimental infection. S mice inoculated with 500,000 live organisms 11c strain* S. typhimurium.

Time following inoculation	Inoculated Mice			Control Mice	
	Condition	Blood p_H	Rectal temperature	Blood p_H	Rectal temperature
20 hours		7.42	37.9 C	7.42	38.0 C
		7.42	37.6 C	7.42	37.9 C
3 days	sick	7.26		7.49	
	sick	7.18		7.37	
	sick	7.18		7.41	
4 days		7.27		7.31	
		7.40		7.47	
		7.43		7.52	
6 days	sick	7.41	36.2 C	7.40	37.8 C
		7.51	37.8 C	7.43	38.4 C
		7.34	37.7 C	7.43	38.1 C
8 days		7.30	36.7 C	7.40	37.8 C
		7.26	38.0 C	7.39	36.7 C
		7.39	37.2 C	7.47	37.4 C
10 days	sick	7.28	37.2 C	7.40	38.0 C
	sick	7.31	35.6 C	7.37	37.6 C
12 days		7.53		7.46	
		7.46		7.42	
		7.43		7.46	
14 days		7.40	37.4 C	7.44	37.6 C
		7.49	37.6 C	7.48	38.0 C
16 days		7.40	33.3 C	7.44	36.4 C
		7.46	34.6 C	7.45	36.9 C
	sick	7.50	32.6 C	7.44	37.0 C
18 days		7.45	37.5 C	7.41	38.3 C
		7.49	36.0 C	7.46	37.8 C
		7.66	36.8 C	7.51	38.8 C

TABLE 13.—*Blood p_H of immunized and control mice. Different mice tested at intervals after immunization with heat killed* S. typhimurium.

Mouse strains	Treatment	Mice tested per day	Day 5		Day 8		Day 15		Day 22		Day 29		Day 36		All mice	
			Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
S & RI	Immunized	10	7.396	±0.012	7.340	±0.016	7.416	±0.015	7.416	±0.009	7.400	±0.018	7.446	±0.009	7.402	±0.007
	Control	10	7.391	±0.016	7.353	±0.022	7.437	±0.009	7.385	±0.017	7.400	±0.016	7.412	±0.023	7.396	±0.008
	Difference		+0.005		−0.013		−0.021		+0.031		0.000		+0.034		+0.006	
L & Ba	Immunized	10	7.351	±0.021	7.332	±0.020	7.435	±0.011	7.341	±0.017	7.355	±0.011	7.338	±0.017	7.359	±0.008
	Control	10	7.325	±0.017	7.352	±0.028	7.392	±0.010	7.338	±0.019	7.305	±0.014	7.311	±0.018	7.331	±0.007
	Difference		+0.026		−0.020		+0.043†		+0.003		+0.050*		+0.027		+0.022	
Z	Immunized	5	7.306	±0.026	7.280	±0.016	7.356	±0.029	7.270	±0.026	7.264	±0.029	7.290	±0.026	7.294	±0.013
	Control	5	7.244	±0.038	7.272	±0.038	7.286	±0.030	7.216	±0.028	7.284	±0.010	7.300	±0.024	7.267	±0.012
	Difference		+0.062		+0.008		+0.070		+0.054		−0.020		−0.010		+0.027	

* Significant beyond the 0.05 level.
† Significant beyond the 0.01 level.

mean temperature of 36.5 C, contrasted with a mean of 37.7 C for controls. The data of table 12 show that the temperature effect is independent of p_H. The lowered temperature persisted even after the p_H had returned to or exceeded that of the controls.

Over a fairly wide dosage range the disease usually runs its course in approximately 15 days in resistant strains. Antibodies are not usually detectable by agglutination tests before that time. It will be observed in figure 5 that the p_H of inoculated mice sampled on the 12th to 16th day was normal and that mice sampled on the 18th day had a p_H above that of the controls. If this result is indicative of a real physiologic change, an important prediction stems from it —namely, immunization will raise blood p_H. This has been tested experimentally.

Changes in blood p_H with immunization

Mice of the various strains were immunized by two intraperitoneal inoculations spaced 10 days apart, each with 2,000,000 heat-killed organisms of the 11c strain of *S. typhimurium*. At 5, 8, 15, 22, 29, and 36 days after the second immunization, 5 immunized and 5 control mice from each strain were p_H tested. Different mice were used each time. Since S and RI and L and Ba strains showed essentially the same picture, the data have been combined in table 13.

The data presented in table 13 lend support to the view that immunization is accompanied by a shift in blood p_H toward greater alkalinity. Statistically the differences are small. Only 2 of 18 blood p_H comparisons between immunized and control mice were statistically significant, compared to an expectation of 1 in 20 on a basis of pure chance. Both significant differences, however, were in the susceptible L and Ba group in which the p_H of immunized

mice exceeded that of the controls on 5 out of 6 days of test. The mean p_H values of 7.435 for immunized susceptibles (L&Ba) and 7.356 for immunized Z on the 15th day after immunization approach the upper limits of normal variation for those particular mouse strains. Agglutination tests were run on blood collected on the 8th, 15th and 22nd day after immunization. Accurate measurement of titer was not obtained but agglutinins were clearly demonstrable on the 15th and 22nd day after immunization and not on the 8th day. Since the rise in p_H roughly corresponds with the appearance of immune bodies and persists for some time, it may be associated with some change in the protein buffers of the serum.

Changes in blood p_H with feeding

Most attempts to alter blood p_H experimentally result in a transitory type of change which is difficult to interpret. For example, in the present study, injection of 0.5 cc of 0.75% ammonium chloride into the lateral tail vein of mice resulted in a fall of 0.10 p_H within an hour, followed by a rise in 20 hours to as much as 0.20 p_H above the control sibs. The same type of effect in humans was reported by Shock and Hastings (1935). In view of these results it was decided to test the effect on p_H of a diet of carrots, which are high in alkaline ash content. Sherman and Gettler's (1912) analysis for carrots shows a base excess of 10.82 cc of a 1*N* solution per 100 g, compared to an acid excess of 5.95 cc for corn and 9.66 to 12.39 for wheat.

It was appreciated that the low protein level (1%) of a diet consisting solely of carrots, compared to the control diet of 16% protein, would likely produce secondary effects, so that the possible significance of p_H changes must be interpreted with extreme caution. However, the findings of Bischoff et al. (1934) that neither acid nor alkaline ash foods would induce changes in blood p_H in humans suggested that only drastic steps offered any hope of producing lasting p_H changes.

Twenty-five sib pairs from each of the S, RI, L and Ba strains were used for the experiment. In addition 11 sib pairs of F_1 (S × Ba) were included. The groups were fed once a day,—sliced carrots for one group, grain for the controls. The grain ration consisted of 53.2% ground corn, 8.51% linseed oil meal, 5.3 % tankage, 2.1% alfalfa leaf meal, 21.3% ground whole wheat, 5.3% powdered buttermilk, 1.6% ground oyster shell, 0.5% salt and 1.1% cod liver oil. Six days after commencement of the experiment 4 sib pairs from each strain were p_H tested. Results are shown in table 14. The mean p_H of the carrot fed mice was higher for all strains, ranging from an increase of 0.026 to 0.085 p_H. No correlation between the typhoid resistance level of the mouse strains and the shift in p_H due to the treatment is apparent. All carrot-fed mice lost weight, the mean losses ranging from 1 g in the S × Ba mice to 3 g in the Ba group. There were 5 deaths in each of the S and Ba carrot-fed groups.

TABLE 14.—*Effect of a carrot diet on blood p_H of sib pairs of RI, S, S×Ba, L and Ba mice. Four sib pairs from each strain tested 6 days after commencement of carrot feeding.*

Mouse strain	Number of sib pairs	Mean blood p_H		Difference
		Carrot group	Grain group	
S	4	7.495	7.415	+0.080
RI	4	7.502	7.472	+0.030
S×Ba	4	7.448	7.420	+0.028
L	4	7.497	7.412	+0.085
Ba	4	7.377	7.342	+0.035
	20	7.464	7.412	+0.052*

* Significant at the 0.05 level.

At this point the S, Ba and S × Ba mice were saved for a switch-over experiment. The RI and L groups, in

which no deaths had occurred, were continued on the same schedule as before, except that a small feeding of grain was given to the carrot group once a week. In addition, the carrot ration was increased by feeding twice a day. This resulted in fresh edible portions being available to the mice at all times.

TABLE 15.—*Distribution of deaths of carrot fed and control RI and L sib pairs inoculated with 11c strain* S. typhimurium. *RI dose, 2,000,000; L dose, 200,000 live organisms.*

Strain of mice	Treatment	Days to death							Alive	Total	Percent survivors
		3–5	6–8	9–11	12–14	15–17	18–20	21–			
RI	Carrots						4	1	16	21	76
	Control	1	1		4	1	1	1	12	21	57
L	Carrots				5		5		10	20	50
	Control		2	2	3	2			11	20	55

The RI and L mice were isolated and inoculated with live organisms of the 11c strain of *S. typhimurium*. Dosages were 2,000,000 for the RI and 200,000 for the L strain. Deaths were recorded over a 21-day period. On a priori grounds a reduction in resistance was rather expected on the low protein carrot diet. It was therefore a surprise to find that the carrot fed mice in the RI strain did not start dying until the 20th day, by which time 7 out of 21 diet controls had already died. A similar, though less extreme picture, was found for the susceptible L strain (table 15). The altered reaction to experimental infection brought about by carrot feeding may be related to the high vitamin A value of carrots, since vitamin A is known to influence the resistance of epitheliel tissue to the entrance of infective organisms. The fact that blood p_H was at the same time shifted toward increased alkalinity suggests that both enhanced resistance and the p_H change may be associated with a common metabolic change.

Most but not all studies on the effect of nutrition on the reaction of laboratory animals to disease indicate a lowering of resistance on "deficient" diets. The work of Robertson and Doyle (1936), Watson (1937) and Webster and Pritchett (1924) support this view. Foster et al. (1944), on the other hand, found increased resistance to the Lansing strain of poliomyelitis in mice fed either a diet deficient in vitamin B_1 or a normal diet but with restricted intake. The effect was to delay the action of the virus rather than prevent it. Sprunt (1942) found that rabbits on a deficient or starvation diet were more resistant to vaccinia virus. Evidently the effect of a given diet on resistance to a particular disease is not always predictable in advance.

The S, Ba and S × Ba mice which had been saved for further experimentation were all fed grain for a month, during which time the groups which had been getting carrots regained their lost weight. The groups were then switched over so that mice which had been getting carrots received grain, and mice which had been getting grain received carrots. At 7, 10 and 12 days after commencement of the trial, blood samples from different sib pairs were p_H tested. Since there was no difference between days, the data were combined.

There were no significant differences in p_H between carrot- and grain-fed mice in either S or S×Ba groups with 13 and 5 sib pairs, respectively. In the Ba strain the mean p_H of the carrot group for 12 sib pairs exceeded the mean of the grain fed group by 0.046 p_H, which is significant beyond the 0.01 level. Tested 10 days after return to the normal diet, the same mice from the carrot group had

a p_H of 7.372, compared to p_H 7.381 for the controls, indicating a recovery of the normal blood p_H.

Since there was a tendency for a higher p_H in the more adversely affected mice on the carrot diet, the possibility arose that the effect might be due to starvation and not the alkaline effect of carrots. To test this, 25 S sib pairs and 25 Ba sib pairs were split into two groups. In one group the regular ration was fed ad libitum; in the other the intake was restricted. Water was available at all times to both groups. The treatment was so severe that when the experiment was terminated after 21 days, 3 out of 25 S mice and 18 out of 25 Ba mice had died in the restricted group, contrasted to no deaths in the controls. The susceptible Ba mice were less able to stand up under the treatment than the resistant S strain. Blood p_H was tested 6, 18 and 21 days after the beginning of the experiment. The differences between means (restricted minus ad libitum groups) were not significant, with values of -0.019 p_H in S and -0.016 p_H in Ba groups. It may therefore be concluded that the elevation of blood p_H by carrot feeding in the previous experiment was not due to starvation effect.

TABLE 16.—*Blood p_H of F_2 mice (S×Ba, S×Z, Ba×Z) and subsequent survival from inoculation with* S. typhimurium, *11c strain, 200,000 live organisms.*

	Alive		Dead		Mean survival days	Correlation p_H and survival	Total mice	Percent survival
	Number of mice	Mean blood p_H	Number of mice	Mean blood p_H				
F_2(S×Ba)	46	7.411	24	7.384	7.2	+0.139	70	65.7
F_2(S×Z)	31	7.335	4	7.345	8.8		35	88.6
F_2(Ba×Z)	19	7.318	28	7.310	7.1	+0.107	47	40.4

Blood p_H and genetic resistance

Since differences between mouse strains in blood p_H have been shown to have a genetic basis, it should be possible in time to isolate high and low p_H strains from a random bred group by selective breeding. A test of such strains for disease resistance would indicate the contribution of p_H to resistance level. A somewhat similar though less critical test for association between p_H and resistance level was performed using F_2 mice from the various crosses. An F_2 population of mice from an original cross between a high p_H, high resistance strain and low p_H, low resistance strain will segregate for both phenotypes. If high p_H and high resistance are related in some way, they should be found together more often than separated in an F_2 from such a cross.

F_2 mice were p_H tested and approximately one month later inoculated with 200,000 live organisms of 11c strain *S. typhimurium*. Deaths were recorded over a 21-day period. The results presented in table 16 show that the mean blood p_H of survivors from the S × Ba cross exceeded that of mice which died by 0.027 (p approximately 0.10) and also show that in the Z (intermediate resistance, low p_H) F_2 crosses there were virtually no corresponding differences in p_H. The correlation coefficients for p_H and day of death for the 24 (S × Ba) F_2 (r = 0.139) and 28 (Ba × Z) F_2 (r = 0.107) were positive but not statistically significant.

The evidence already presented for a correlation between high blood p_H and resistance finds some support in the results of this experiment. The picture is, in fact, of the type to be expected on the assumption that genetic resistance to mouse typhoid is due to a fortuitous

combination of a number of gene-controlled mechanisms, the contribution of any one probably being small.

Although, with the numbers used, the small differences in p_H between survivors and mice which died may have been due to chance, there are several things which argue against this possibility. In the first place, the differences in means and the correlation coefficients, both of which indicate a positive correlation between p_H and resistance, are independent estimates. Secondly, larger differences in p_H between survivors and non-survivors in the S × Ba F_2 contrasted to the Z crosses were predicted, as the S and Ba strains are more widely separated in resistance level. Finally, since per cent survival is a crude measure of resistance and variations in p_H are to a large extent influenced by environment, the tendency may be to underestimate the biological significance of p_H in the resistance pattern.

DISCUSSION

The present study has shown that about 40% of the variations in blood p_H in mice can be accounted for by genetic differences between the 5 inbred strains. Each inbred strain is characterized by its particular blood p_H level. This means that differences in blood p_H in mice, which correspond with those ascribed to "individual variation" in human studies, have been segregated into pure breeding strains in the present material.

There are several possible ways in which gene-influenced characters may be so fractionated into distinct inbred lines:

1. Inbreeding without selection in a variable population will produce different lines due to chance fixation of different genes.

2. Inbreeding with selection will fix "high" or "low" lines for the particular character selected.

3. Inbreeding with selection for one character will fix other characters which may bear a causal relationship to the one selected.

Since there has not been artificial selection for blood p_H in mice, the fixation of genes which influence p_H must have come about by chance or as a result of selection for disease resistance or susceptibility. Both alternatives are possible. The S and RI inbred strains, which differ in a number of characteristics such as size and fertility, were formed in different laboratories by selection for resistance to mouse typhoid. In the process the blood p_H of both strains has been fixed at a mean of 7.43. The L strain, which was developed by selection for susceptibility, and the Ba strain, in which susceptibility has been fixed by inbreeding without selection, are both characterized by a mean blood p_H of 7.36. Statistically the differences between resistant and susceptible strains with respect to both resistance and blood p_H are highly significant.

Further evidence for a correlation between resistance and blood p_H was obtained from the results of p_H and resistance tests in a segregating (F_2) population from a resistant (S) by susceptible (Ba) cross. The mean p_H of 7.411 for F_2 mice which subsequently survived artificial infection and the mean of p_H 7.384 for non-survivors approach the high and low resistance parent values of p_H 7.427 and 7.361, respectively. Altogether, it is highly improbable that the correlation between resistance and blood p_H in the 4 strains S, RI, L and Ba is due to either genetic linkages or mere chance.

The character basis of genetic resistance to mouse typhoid is apparently complicated. For example, number of leucocytes (Gowen and Calhoun, 1943) and ability of macrophages to digest phagocytosed bacteria (Oakberg, 1946)

have been shown to influence genetic resistance. Blood p_H, therefore, whether it is a primary mechanism or related to resistance in a secondary way, must be thought of as, at most, only one of a number of mechanisms. The intermediate resistance level of the low p_H Z strain may be interpreted as due to presence in the Z mice of mechanisms other than blood p_H which are lacking in the Ba and L strains.

Some light may be cast on the validity of this assumption if it can be established that Z mice do indeed possess other mechanisms of resistance, and if at the same time it can be shown that they are adversely affected by the disease due to their low p_H. Some evidence has been obtained relevant to these considerations. Weir (1942) found a higher antibody titer in immunized Z mice than in any of the other strains, yet there was no correlation between agglutinin production and resistance when the 6 strains were considered. The data were interpreted as indicating a superiority of the intermediate Z over resistant strains for this particular mechanism. The data of Dr. Oakberg (unpublished) also point to a consistently higher titer in the Z strain.

Concerning a possible relationship between the low p_H and disease pattern in the Z mice, Oakberg's (1946) observation on glycogen storage may be pertinent. In this respect the intermediate Z resembled the susceptibles in that glycogen content of the liver practically disappeared with the onset of morbidity. Evidently infected Z mice lack the ability of the S and RI strains to carry on normal carbohydrate metabolism.

There is evidence in the literature that disturbed carbohydrate metabolism is accompanied by p_H changes. Guest and Rawson (1941) found a correlation between induced p_H changes in the blood of the rat and the stored and mobile carbohydrate. Liver glycogen was found to vary directly with blood p_H in the lower range and blood sugar to vary inversely throughout the p_H range studied.

No good experimental evidence has been obtained concerning the mechanisms of inherited p_H differences in normal mice. It is reasonable to suppose that one or more of the known buffers of the blood are involved. These include the phosphate, bicarbonate and protein buffer systems. It is known that the system composed of carbon dioxide and bicarbonate is especially important in regulating p_H. It is unlikely, however, that this system alone can account for the strain differences, as p_H measurements of arterial blood exceed those for venous blood by the same amount in all strains.

There is some evidence pointing to a relationship between changes in blood p_H and the serum proteins. The slight rise in p_H observed after recovery from infection or following artificial immunization, for example, corresponds with the period in which antibodies are demonstrable in the serum. This would point to an effect of serum globulins since it has been shown that antibodies are specifically modified molecules of globulin. If the p_H rise with immunization should prove to have such a simple basis it might be predicted that animals made hypoproteinemic by a low protein diet and, according to Cannon et al. (1943), with a lessened capacity to produce agglutinins, would have a lowered p_H. A diet consisting solely of carrots, which results in a fall in the concentration of serum proteins (Bloomfield, 1934), might then be expected to lower the blood p_H. Actually the p_H was raised by carrot feeding. This may have been due to some alteration in the protein buffer system, to the alkaline ash effect of carrots or some other effect.

Surprisingly, carrot-fed mice proved more resistant to infection with *S. typhimurium* than sibs on a so-called complete diet. Carrot-fed RI and L mice outlived diet control litter mates by 7 and 3 days, respectively, in a 21-day test.

Evidence has been presented which shows that p_H is independent of body temperature in both normal and diseased mice. Body temperature, in turn, is apparently not related to resistance (Weir, 1947). Further, unpublished data cited by Gowen and Calhoun (1943) from experiments in which external temperatures were varied from 50 to 100 F, show no effect of room temperature on resistance.

SUMMARY

Blood p_H was determined for 5 inbred strains of mice by means of a glass electrode. The S and RI strains, which are characterized by a high level of genetic resistance to mouse typhoid, were found to have mean p_H values of 7.427 and 7.434. The means for susceptible strains were L, 7.355 and Ba, 7.361. For intermediate genetic resistant Z mice the mean p_H was 7.285. Differences between strains accounted for approximately 40% of the variation in blood p_H.

Blood p_H of mice kept at 32 C exceeded that of sibs held at 24 C by 0.028 to 0.042 p_H. No correlation was found between rectal temperature and p_H in any of the strains. The p_H of arterial blood exceeded that of venous blood by 0.115, with no difference between strains. Sex, age and body size had no significant effect on p_H.

Blood p_H determinations were made on F_1 and F_2 mice of S×Ba, S×Z and Ba×Z crosses. The p_H of S×Ba F_1 mice was intermediate between the parents and the parental extremes were recovered in F_2. The S×Z F_1 exceeded the midparent value by 0.033, and the Ba×Z F_1 exceeded its midparent value by 0.037. Both differences are significant. The F_1 range exceeded the F_2 in both S×Z and Ba×Z crosses. The standard deviations were Z, 0.078; S×Z F_1, 0.078; F_2, 0.070; Ba×Z F_1, 0.082 and F_2, 0.073.

Inoculation of S mice with 500,000 live organisms of *S. typhimurium* resulted in an acidosis with p_H 7.21, contrasted to 7.42 for controls on the third day after inoculation. The p_H returned to normal by 12 days after inoculation. By the 18th day the p_H exceeded controls by 0.07.

Immunization with heat-killed organisms resulted in a rise in p_H in all strains of mice. The rise corresponded in time of appearance with the rise in antibody titer. Increases in the mean p_H of the susceptible group of 0.043 and 0.050 p_H above controls, 15 and 29 days after immunization, were statistically significant.

Mice of the 5 strains on a diet consisting solely of carrots had a significantly higher mean blood p_H (0.03 to 0.08) than control sibs. Inoculated carrot-fed mice of RI and L strains outlived controls in a 21-day test period by 7 days in the RI and 3 days in the L strain.

S and Ba mice on a restricted intake of grain did not differ in blood p_H from controls fed ad libitum.

F_2 crosses which had previously been p_H tested were inoculated with 200,000 mouse typhoid organisms. Survivors among the F_2 S×Ba mice (resistant by susceptible) had a blood p_H of 7.411, contrasted to 7.384 for mice which died.

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CYSTEINE AND RELATED COMPOUNDS IN THE GROWTH OF THE YEASTLIKE PHASE OF *HISTOPLASMA CAPSULATUM*

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Histoplasma capsulatum Darling, the etiologic agent of histoplasmosis, has two morphologically distinct but reversible growth phases. (1) The yeastlike phase, which is the only one present in the tissues of man or animals, has been cultured principally on rabbit blood or serum agars in sealed tubes at 37 C since the original report of DeMonbreun.[1] Campbell[2] also reported its growth on Francis' glucose cystine blood agar, while Salvin[3] succeeded in obtaining excellent growth of this phase in a semifluid peptone medium. (2) The mycelial phase, which grows more readily "in vitro" than the yeastlike phase, has been cultured at room temperature on a wide variety of mediums, such as dextrose agar,[1] brain agar, meat infusion agar,[4] and an asparagin-glucose broth.[5]

The yeastlike phase does not develop on all mediums that support the mycelial phase, even if such environmental factors as temperature and hydrogen-ion concentration are at an optimum. DeMonbreun,[1] for example, reported that such mediums as glucose agar, Sabouraud's agar, potato glucose agar, or gelatin would promote growth of only the mycelial and not the yeastlike phase.

The presence of two such distinct phases within a single species produced questions as to (a) whether the two phases had profoundly different nutritional requirements, (b) whether, other factors being equal, changes in the contents of the medium could produce a conversion of one phase to the other, and (c) what enzymatic processes dominated the growth of each phase. Accordingly, experiments were initiated to determine what specific nutritional factors were essential and limiting for each phase. This paper reports investigations on the nitrogen and growth-factor demands of both the mycelial and yeastlike phases.

MATERIALS AND METHODS

The 8 strains of *H. capsulatum* used were obtained from the collection maintained in this laboratory, although they were originally isolated from the following sources:

Strain number	Source
6502	man[1]
6510	man[6]
6513	man
6515	dog[7]
6518	dog[8]

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1. DeMonbreun, W. A. 1934, The cultivation and cultural characteristics of Darling's *Histoplasma capsulatum*. Am. J. Trop. Med. **14:** 93–125.
2. Campbell, C. C. 1947, Reverting *Histoplasma capsulatum* to the yeast phase. J. Bact. **54:** 263–264.
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Strain number	Source
6519	man[9]
6521	dog[10]
6523	man[11]

The mycelial phase was maintained on Sabouraud's agar slants at 25 C. The yeastlike phase, after isolation from the spleens or kidneys of mice experimentally infected with a suspension of conidia in saline, was maintained in sealed tubes on 20% rabbit blood agar at 37 C.

Each of the following experiments was conducted in duplicate with a minimum of 6 of the above strains and was repeated at least once. The basal medium employed for the yeastlike phase was as follows:

sodium chloride	5.0 g
disodium phosphate (anhydrous)	2.5 g
glucose	2.0 g
magnesium sulfate · $7H_2O$	0.25 g
calcium chloride	0.25 g
agar	1.5 g
biotin	50 gamma
folic acid	50 gamma
inositol	10 mg
niacin	1 mg
calcium pantothenate	1 mg
p-aminobenzoic acid	10 mg
pyridoxine	1 mg
riboflavin	1 mg
thiamin	1 mg
water to make	1000 ml

When a single nitrogen-containing compound was used, 1.8 g/L were added to the medium. When a mixture of non-sulfur-containing amino acids was used as the source of nitrogen, 10 mg of each of the following crystalline amino acids was included in the medium (Synthetic amino acids were employed where possible to avoid possible cysteine, cystine or methionine contamination): glycine, dl-alanine, dl-serine, dl-threonine, dl-valine, dl-leucine, dl-isoleucine, dl-phenylalanine, l-tyrosine, dl-tryptophane, l-proline, dl-aspartic acid, l(+)glutamic acid, l-histidine hydrochloride monohydrate, l(+)arginine, and l(+)lysine. It was found that an acid hydrolysate of casein was not low enough in cystine content to permit its use.

In all the experiments in which growth-factor requirements were studied, the agar was washed for a minimum of 5 days in running water, then rinsed in distilled water and dried. The medium, after adjustment to p_H 7.3, was generally dispensed in 8 to 10 ml quantities in test tubes, and after inoculation with the fungus was incubated either at 37 C in sealed tubes for 3 weeks for the yeastlike phase, or for 6 weeks at 25 C for the mycelial phase. All growth at 37 C was examined microscopically to determine its character,—mycelial or yeastlike. When the number of cells was estimated, a Levy hemocytometer or a Fisher electrophotometer was employed.

TABLE 1.—*Growth of the yeastlike phase of* H. capsulatum *in semifluid peptone medium[3] for two weeks at 37 C after inoculation with different quantities of the yeastlike cells.*

Size of inoculum (thousands of cells per ml of total medium)	Thousands of cells/ml	
	No CO_2	7% CO_2
1,000	415,000	422,000
200	565,000	562,000
40	130,000	390,000
8	no growth	182,500
1.6	no growth	no growth

The inoculum was either (a) a loopful of a dense washed suspension of microconidia, macroconidia and mycelial fragments in saline, or (b) 0.05 ml of a washed suspension of 100,000,000 to 200,000,000 yeastlike cells per ml. Such a high number of the yeastlike cells was necessary for growth, as seen in table 1. An increase of the carbon dioxide in the environment to 7% during the first 72 hours of growth affected very slightly the necessary size of the inoculum (table 1).

EXPERIMENTS AND RESULTS

Nitrogen requirements of the yeastlike phase.—Six strains were inoculated into tubes in which 1.8 g/L of one of the following constituted the sole source of nitrogen: purified casamino acids* plus cystine and tryptophane; ammonium

* Casamino acids consist of the acid hydrolysate of purified casein.

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nitrate; a mixture of equal quantities of adenine, xanthine, hypoxanthine and guanine; uracil; and proteose peptone. After incubation, extensive growth was evident only in the tubes with the casamino acids or the proteose peptone.

Further attempts were made to grow the fungus in mediums with potassium nitrate, ammonium sulfate, ammonium nitrate, urea, or acetamide. However, none of these non-amino-acid nitrogen compounds supported the development of the yeastlike phase.

Accordingly, 8 strains were inoculated into tubes from each of which a different one of the following 20 amino acids was omitted: glycine, dl-alanine, dl-serine, dl-threonine, dl-valine, dl-leucine, dl-isoleucine, dl-phenyl alanine, l-tyrosine, l(+)cysteine hydrochloride, l-cystine, dl-methionine, dl-tryptophane, l-proline, l-hydroxyproline, dl-aspartic acid, l(+) glutamic acid, l-histidine, l(+)arginine, and l(+)lysine. The yeastlike phase developed extensively in all these mediums, with little difference existing between the tubes and with the actual counts averaging about 50,000,000 cells per ml of medium. Thus, no single amino acid may be considered as essential to the growth of the organism in the medium employed.

Tubes of the basal medium were prepared in each of which 1.8 g/L of a different single amino acid were included. After inoculation with 6 strains of the yeastlike phase and incubation for three weeks at 37 C, good growth of the yeastlike phase, i.e., 10,000,000 to 14,000,000 cells per ml, occurred in only two of the 20 amino acid mediums,—namely, the ones with cysteine and cystine. The others showed either no growth or development of the mycelium to the exclusion of the yeastlike cells. For example, alanine, which is similar to cysteine in molecular structure except for the absence of a sulfhydryl group from the β-carbon atom, and serine, which is like cysteine except for the substitution of a hydroxyl (-OH) for the sulfhydryl (-SH) group, supported abundant growth of the mycelium only. No growth of any kind occurred in the medium with methionine or methyl cysteine alone.

The yeastlike phase was inoculated into mediums with 1.8 g/L of one of several other nitrogen-containing compounds, such as p-aminobenzoic acid, methylamine, creatine, betain, choline, glucosamine, histamine, asparagine, ornithine, citrulline, uric acid, allantoin, and glutathione. Extensive development of the yeastlike phase appeared only in one,—the medium with glutathione, which is a tripeptide composed of glutamic acid, cysteine, and glycine.

At one point during the course of this study, the yeastlike cells of strain 6523 grew well in arginine and the related compounds, ornithine and citrulline, reaching a concentration of 8,000,000 cells per ml. However, the faculty to utilize these polyamino carboxylic acids was quite transitory, for after two transfers in a medium in which arginine was the sole nitrogen compound, growth no longer occurred.

When a medium with 16 non-sulfur-containing amino acids was prepared, inoculation with the yeastlike phase and incubation at 37 C for three weeks produced mycelial growth alone in the 8 strains tested (fig. 1). However, when 0.002 *M* of either l(+) cysteine hydrochloride, l-cystine, or dl-methionine was added to the medium, the yeastlike phase developed luxuriantly (fig. 2). Apparently, the sulfide (-S-) or sulfhydryl radical is necessary for the growth of the yeast-like phase, for its omission from the medium (as in the substitution of alanine for cysteine in the medium) or its replacement (as in the substitution of serine for cysteine) resulted in the development of the mycelial phase.

The following compounds in 0.002 *M* amounts were then tested for their ability to induce growth of the yeastlike phase against 6 strains of *H. capsulatum* at 37 C in a medium with 16 non-sulfur-containing amino acids as the nitrogen source (the magnesium sulfate in the basal medium was replaced by the equimolar quantity of magnesium chloride): sodium persulfate, sodium sulfate, sodium bisulfite, sodium hydrosulfite, sodium sulfide, sodium hydrosulfide, cysteine, cystine, methionine, sodium thioglycolate, methyl cysteine, homocysteine,* thiozolidine carboxylic acid,* cysteic acid,† diethylthiourea, glutathione, and diglycylcystine.* Ammonium sulfate or nitrate was not used in the medium as the nitrogen source, because the resulting growth of the yeastlike phase at 37 C was slight and included some mycelium.

As seen in table 2, growth of the yeastlike phase occurred only in those mediums which had compounds containing either a sulfide or a sulfhydryl group None of the compounds, organic or inorganic, containing oxidized sulfur promoted growth of the yeastlike phase. Also, those mediums with the reduced sulfur in inorganic form did not stimulate development of the yeastlike cells. Where the activity of the sufide or sulfhydryl group was seemingly hindered by the complexity of the molecule, either the yeastlike phase was completely inhibited in its development (e.g., in

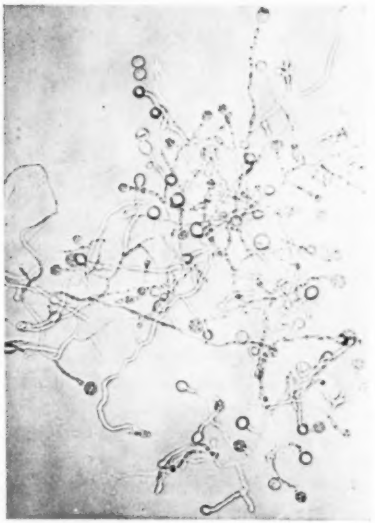

FIG. 1.—Three weeks growth of *H. capsulatum* in a medium with 16 non-sulfur-containing amino acids at 37 C with 5,000,000 washed cells of the yeastlike phase as the inoculum. (Note growth of mycelium and macroconidia and complete absence of the yeastlike phase.) ×250.

FIG. 2.—Three weeks growth of *H. capsulatum* in a medium with 16 non-sulfur-containing amino acids plus 0.002 M cysteine at 37 C with 5,000,000 washed cells of the yeastlike phase as the inoculum. (Note growth of yeastlike cells exclusively.) ×250.

* Compounds obtained through the courtesy of Dr. M. X. Sullivan.

† Compound synthesized by Dr. E. L. May.

mediums with thiozolidine carboxylic acid), or both mycelium and yeastlike cells appeared (e.g., in mediums with glutathione and diglycylcystine as the sulfur source). Of interest is the fact that sodium thioglycolate, which is organic and contains a sulfhydryl group but has no amino group, supported not only good growth of the yeastlike phase but also a moderate amount of mycelium. Methionine and methyl cysteine, which were not available to the fungus as sources of nitrogen when no other nitrogen-bearing compounds were in the medium, did serve to overcome the specific sulfur block and promote growth of the yeastlike phase.

The comparative activities of cysteine, cystine, methionine, cysteic acid, sodium hydrosulfide, sodium thioglycolate, and glutathione in inducing the growth of the yeastlike phase were then determined. One strain (no. 6521) of *H. capsulatum* was inoculated into the basal medium containing (a) 16 non-sulfur-containing amino acids as the source of nitrogen and (b) varying quantities of one of the above sulfur compounds. The resulting growth (when present) was examined periodically to determine its character and extent (table 3). During the first few days of incubation, growth in the mediums with organic sulfide or hydrosulfide was yeastlike. However, in those mediums containing only 10^{-5} *M* of organic -S- or -SH, growth then became mycelial, apparently as the small amount of sulfur was metabolized. At 10^{-4} *M* of organic -S- or -SH, all growth with the exception of cystine was mycelial at the end of three weeks' incubation. Cystine promoted growth of the yeastlike cells at this low concentration probably because it contained twice as much sulfur per mole as each of the other compounds tested. Regardless of the concentration, glutathione and sodium

TABLE 2.—*Growth of 6 strains of* Histoplasma capsulatum *in a medium with 16 non-sulfur-containing amino acids as the nitrogen source and with different compounds as the source of sulfur (after 3 weeks incubation at 37 C).*

Sulfur-containing compound	Extent of growth of the yeast-like phase	Type of growth
$Na_2S_2O_4$	−	myc
Na_2SO_4	−	myc
$NaHSO_4$	−	myc
$Na_2S_2O_4$	−	myc
Na_2S	−	−
NaHS	tr	myc, yp*
l(+)cysteine hydrochloride	+	yp
l-cystine	+	yp
dl-methionine	+	yp
sodium thioglycolate	+	yp, myc
cystine amine	−	−
methyl cysteine	+	yp
dl-homocysteine	+	yp
cysteic acid	−	myc
thiozolidine carboxylic acid	−	−
diethylthiourea	−	−
glutathione	+	yp, myc
diglycylcystine	+	yp, myc
control (no sulfur)	−	myc

− indicates no growth.
tr indicates a trace of growth.
+ indicates good growth.
myc indicates mycelial growth.
yp indicates yeastlike growth.
* Growth was almost entirely mycelial.

TABLE 3.—*Growth of strain 6,521 of* Histoplasma capsulatum *at 37 C in a medium with 16 non-sulfur-containing amino acids as the nitrogen source and with different compounds as the source of sulfur (after three weeks' incubation at 37 C).*

Sulfur-containing compound	Molarity of sulfur-containing compound in culture medium					
	10^{-3}	10^{-4}	5×10^{-4}	10^{-3}	2×10^{-3}	5×10^{-3}
l(+) cysteine	myc	myc	yp	yp	yp	yp
l-cystine	myc	yp	yp	yp	yp	yp
cysteic acid	myc	myc	myc	myc	myc	myc
glutathione	myc	myc	yp, myc	yp, myc	yp, myc	yp, myc
methionine	myc	myc	yp	yp	yp	yp
sulfhydrate	myc	myc	myc	myc	—	—
thioglycolate	myc	myc	yp, myc	yp, myc	yp, myc	yp, myc
(control) serine	myc	myc	myc	myc	myc	myc

yp indicates yeastlike growth.
myc indicates mycelial growth.
— indicates no growth.

thioglycolate always induced development of mycelium together with the yeastlike cells. The rate of growth and the final concentration of the cells were approximately the same for the compounds cysteine, cystine, glutathione, methionine, and sodium thioglycolate, namely, about 50,000,000 cells per ml of medium. Growth in the mediums with sodium sulfhydrate was primarily mycelial and extremely slight.

Nitrogen requirements of the mycelium.—Since the yeastlike phase needed reduced sulfur in the medium, the mycelial phase was studied to determine some of its requirements. Six strains were tested at 25 C on mediums in which inorganic nitrogen was the sole source of that element. Growth on mediums with ammonium chloride, ammonium sulfate and ammonium nitrate was luxuriant, but on mediums with potassium nitrate or sodium nitrate was quite limited although present. At no time was mycelial development observed on a medium containing nitrite as the sole source of nitrogen. The mycelium not only did not require any single amino acid for its development but grew well on mediums with a wide variety of organic compounds as the only source of nitrogen, such as asparagine, acetamide, and glucosamine.

When the yeastlike phase was inoculated into a medium with no biotin and with alanine as the only nitrogen compound and then incubated at 37 C for three weeks, the resulting growth was mycelial in character. However, when a suspension of conidia was inoculated into a medium with 50 gamma per L of biotin and with cysteine as the sole source of nitrogen and incubated at 37 C for three weeks, 3 out of 6 strains tested produced some yeastlike cells in the resulting growth.

Growth-factor requirements of the yeastlike phase.—In the previously described mediums in which growth of the yeastlike phase was extensive, 9 growth factors were included, namely, biotin, folic acid, inositol, niacin, p-aminobenzoic acid, calcium pantothenate, pyridoxine, riboflavin, and thiamin. In order to learn whether any single growth factor was necessary for the growth process in the yeastlike phase, a series of tubes were prepared containing 180 mg/L cysteine as the source of nitrogen and with a different one of the 9 growth factors omitted from each tube. After inoculation and incubation for three weeks at 37 C, the yeastlike phase of 8 different strains developed abundantly in all the tubes except the one in which there was no biotin. When another series was set up to each one of which only one of the 9 growth factors was added, good growth appeared only in the mediums with biotin. No significant growth of the yeastlike phase was observed in the presence of the other 8 growth factors, with what was present being mycelial.

TABLE 4.—*Variation in the growth of the yeastlike phase of strain 6,521 of* H. capsulatum *in the presence of different concentrations of biotin (after 21 days of growth at 37 C).*

Concentration of biotin (gamma per L)	Thousands of cells per ml
10	10,000
5	12,500
2	11,500
1	10,000
0.05	225
0.02	250
0.01	175
0.0	50

When the yeastlike cells were inoculated into mediums containing quantities of biotin from 0 to 50 gamma per L and were incubated for three weeks at 37 C, a sharp decrease in the amount of growth of 6 strains occurred in concentrations of biotin in the vicinity of 0.01 to 2 gamma per L (table 4), depending on the strain. Also, when amounts from 10 to 100 gamma per L of either desthio-

biotin (in which two hydrogen atoms have been substituted for the sulfur of biotin) or o-heterobiotin (in which the sulfur in the biotin molecule has been replaced by an oxygen atom) were included in the medium in place of biotin, no extensive growth of the yeastlike phase occurred. Thus, the sulfur in the biotin molecule seemed essential for the development of the yeastlike phase.

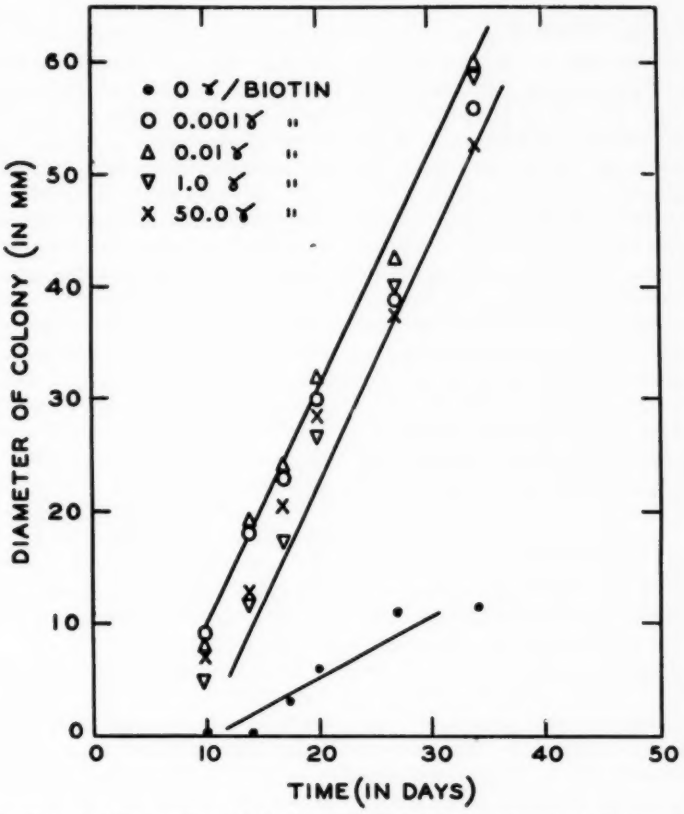

FIG. 3.—Growth of the mycelium of a biotin-requiring strain of *H. capsulatum* in 5 concentrations of biotin.

Growth-factor requirements of the mycelial phase.—With the mycelium having developed so extensively in a medium with 9 growth factors present, experiments were set up to determine whether any single growth factor was necessary. The conidia of 6 strains of *H. capsulatum* were washed off the surfaces of Sabouraud's agar slants and thoroughly washed several times by suspension in saline and centrifugation. A loopful was then inoculated onto the surfaces of mediums containing alanine as the nitrogen source, and only a single one of the following growth factors: adenine, biotin, folic acid, inositol, niacin, p-amino-

benzoic acid, calcium pantothenate, pyridoxine, riboflavin, thiamin and uracil. Controls with all and none of the growth factors were inoculated simultaneously.

After incubation at 25 C for 6 weeks, luxuriant growth of all the strains with the exception of no. 6519 occurred in the mediums with or without any one of the above 11 growth factors. However, the mycelium of strain 6519 developed extensively only in those mediums containing biotin, with merely a trace of mycelium appearing in the mediums with the other growth factors. This need for biotin did not disappear after several transfers over a period of three months. When the mycelium of strain 6519 was inoculated on agar plates with varying amounts of biotin and incubated at 25 C, optimum growth of the mycelium occurred in those plates with as little as 0.001 gamma per L of biotin, although the medium with no biotin had only a trace of growth (fig. 3).

DISCUSSION

Many of the pathogenic fungi have two growth phases: one occurring in the host tissue and in some cases in suitable artificial culture mediums at 37 C; the other "in vitro" at room temperatures. Outstanding in this group are (a) *Blastomyces brasiliensis* and *Blastomyces dermatitidis*, both of which form budding phases at 37 C and mycelial phases at 25 C;[12,13] (b) *Coccidioides immitis*, which forms large endospore-containing sporangia in the tissue and mycelium in artificial culture; and (c) *Phialophora verrucosa* and the other species causing chromoblastomycosis, which appear as subspherical cells or groups of septate cells in the tissue and as mycelium in the culture tube.

Of these *H. capsulatum* is the first shown to have a tissue phase requiring accessory growth factors for development,—namely, biotin and a reduced sulfur group. Possibly because of this need for special growth factors, the tissue phases of *C. immitis* and the *P. verrucosa* group do not occur in artificial culture, although the sporangia of the former have been reported[14] in an anaerobic albumen medium and sporangia-like structures in old agar cultures.[15]

This requirement of the yeastlike phase for a reduced sulfur group was best fulfilled when the -S- or -SH group appeared in an organic molecule of small size, preferably in an amino acid. The position of the -S- in the molecule seemed of slight importance, since cysteine and methionine had equal activity. However, if the molecular size was too great, as in the tripeptides glutathione and diglycylcystine, some mycelium accompanied development of the yeastlike cells. The compound diethylthiourea did not promote growth of the yeastlike phase but actually inhibited it.

The characteristic of the organism to grow (a) as the yeastlike phase in a medium with cysteine as the nitrogen source and (b) as the mycelial phase in a medium without biotin or reduced sulfur was employed for inducing the conversion of one phase to the other. Conidia introduced into a cysteine medium at 37 C developed into yeast-

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like cells without initial passage through a laboratory animal, and the yeastlike cells formed germ tubes and subsequently mycelium in an alanine medium at 37 C. Thus, at a neutral p_H, the substrate plus the temperature is of utmost importance in the growth of the yeastlike phase. This influence of cysteine and related compounds on the yeastlike phase may explain the results of Campbell,[2] who reported conversion of mycelial to the yeastlike phase after repeated transfers on Francis' glucose cystine blood agar.

In 5 of 6 strains studied, the mycelium required no special growth substances. One strain, however, did not show normal growth unless biotin in amounts of 0.001 gamma per L or higher was added to the medium. It is possible that the mycelium of all strains need biotin, but in amounts too low to be detected by the techniques employed.

No strain of the yeastlike phase, on the other hand, developed in the semifluid medium unless a minimum of about 0.1 to 2.0 gamma per L of biotin was added to the medium. This suggests that biotin is an essential part of the system that governs production and growth of the yeastlike phase.

SUMMARY

No single amino acid was necessary for the growth of the mycelial and yeastlike phases of *H. capsulatum*. The mycelial phase also grew with ammonium as the sole nitrogen source.

A sulfide or sulfhydryl group in a small organic molecule, preferably an amino acid, was essential for the growth of the yeastlike phase.

Biotin was necessary for the development of the yeastlike cells, but with one exception had no effect on mycelial growth.

The mycelial phase was changed to the yeastlike phase, and vice versa, at constant temperature (37 C) by varying the contents of the culture medium.

STUDIES ON THE SUPPRESSION OF FUNGUS GROWTH BY THIOUREA. III. THE EFFECTS OF PROTEIN FRACTIONS, AMINO ACIDS, AND THIOL COMPOUNDS

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In previous communications it has been shown that the inhibition of the growth of fungi by thiourea and certain derivatives could be cancelled by adding blood, serum, or plasma to dextrose-Sabouraud culture mediums. This effect appeared to be associated in some way with colloids, since it was either absent or greatly diminished in biological fluids which did not contain proteins and lipids. Thus, in the presence of spinal fluid, urine, or of an ultrafiltrate of serum, thiourea partially or completely suppressed the growth of fungi.[1,2] In the experiments reported in this paper attempts have been made to define the effects of added protein fractions, amino acids, and of thiol compounds, as well as certain other substances, on the suppression of the growth of fungi by thiourea.

MATERIALS AND METHODS

The fungistatic activity of thiourea was studied by the same procedure in three series of experiments. Dextrose-Sabouraud mediums containing added 0.9% saline, or solutions of compounds or substances whose effect on thiourea was to be determined, were inoculated usually in triplicate with *Cryptococcus hominis*. For the latter a suspension of a 2 to 4 day culture of the organism quantitated according to the McFarland scale was diluted before use. To one-half of the pour plates in each group a solution of thiourea in 0.9% saline was introduced in amounts sufficient to obtain a final concentration of 50 mg per cent. Saline alone was added to the remainder. Except as indicated in the footnote to table 2, all solutions were sterilized prior to use by autoclaving or by passage through a Chamberland, Seitz, or Berkefeld filter.

In the initial studies (table 1) human serum or plasma protein fractions separated by means of ammonium sulphate were tested.[3,4] In the fractionation an equal volume of saturated ammonium sulphate was added slowly at 4 C to serum or plasma, precipitating out globulins. The supernatant fluid, containing chiefly albumins, was dialyzed at this low temperature in several changes of distilled water, with or without rotation of the cellophane sac. The precipitate of globulins was redissolved in distilled water and similarly dialyzed. The euglobulins were precipitated out during this process, leaving pseudoglobulins in solution. The euglobulins were then redissolved in saline. In the second set of experiments (table 2) the effects of various Cohn fractions‡ of the plasma proteins[5] on the fungistatic activity of thiourea were examined. The com-

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‡ These fractions were kindly supplied by the Department of Physical Chemistry of the Harvard Medical School. The products of plasma fractionation were developed from blood collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

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TABLE 1.—*Effect of protein fractions obtained by ammonium sulphate separation on the fungistatic activity of thiourea.*

Fraction	Concentration in medium	Inoculum (McFarland)*	Incubation at 37 C (days)	Growth on dextrose-Sabouraud medium: Without added protein fraction		With added protein fraction	
				No S-urea	S-urea (50 mg %)	No S-urea	S-urea (50 mg %)
Albumin	400	1.25	25	444444	000000	444	000
	1200	1.25	25			444	000
Albumin	400	0.85	3	444444	000000	444	111
Albumin	400	1.0	14	444	000	444	012
Albumin	400	0.75	13	444	000	444	000
Albumin	400	1.5	13	444	000	444	000
Albumin	400	3.0	13	443	000	433	000
Albumin	400	0.75	24	332333	000000	444	000
Albumin	400	1.5	6	444444	000000	444	001
Pseudo- and euglobulins	60	1.5	10	444	000	444	333
	120	1.5	10			444	333
Pseudo- and euglobulins	60	1.5	6	444	000	444	000
	120	1.5	6			444	000
Pseudo- and euglobulins	60	1.5	14	444	000	034	000
	120	1.5	14			442	310
Pseudo- and euglobulins	60	1.5	14	444	000	444	000
	120	1.5	14			444	000
Pseudo- and euglobulins	60	1.5	11	444	000	444	000
	120	1.5	11			44	21
Pseudo- and euglobulins	60	1.25	23	444444	000000	43	00
Pseudo- and euglobulins	60	0.75	24	333333	000000	444	000
Pseudoglobulins	320	0.5	7	444444	000000	444	321
	650	0.5	7			444	333
Pseudoglobulins	320	5.0	10	444444	000000	444	433
	650	5.0	10			444	444
Pseudoglobulins	250	1.2	16	444444	000000	444	444
Pseudoglobulins	250	1.5	20	444444	000000	444	444
Pseudoglobulins	320	1.5	25	444444	000000	444	000
Pseudoglobulins	320	1.5	7	444444	000000	444	444
Euglobulins	60	0.5	10	444444	000000	444	000
	120	0.5	10			444	000
Euglobulins	60	5.0	10	444444	000000	444	000
	120	5.0	10			444	000
Euglobulins	60	1.2	16	444444	000000	444	003
Euglobulins	60	1.5	28	444444	000000	444	443
Euglobulins	60	0.85	25	444444	000000	222	000
Euglobulins	60	1.5	6	444444	000000	444	222

* Diluted 1:100 with 0.9% saline solution before use.

TABLE 2.—*Failure of Cohn fractions to cancel thiourea fungistatic activity.*

Fraction	Concentration in medium (mg %)	Inoculum (McFarland)*	Incubation at 37 C (days)	Growth on dextrose-Sabouraud medium: Without added protein fraction		With added protein fraction	
				No S-urea	S-urea (50 mg %)	No S-urea	S-urea (50 mg %)
I		0.5	14	222233	000000	33	0
II†	60	1.5	8	444	000	01244	000000
	120	1.5	8			444	0000
II	60	0.5	13	444	000	444	000
	120	0.5	13			444	000
II+III	90	1.75	13	444	000	44	000
	180	1.75	13			444	000
II+III†	60	1.5	8	444	000	444444	000000
	120	1.5	8			434	000000
III-0	40	1.5	8	444	000	444431	000000
	80	1.5	8			44	00
III-2	70	0.5	14	222233	000000	33	00
IV-1	50	0.5	14	222233	000000	32	00
IV-1	50	1.75	22	444444	000000	444	000
IV-4	45	0.5	14	222233	000000	32	00
IV-4	45	1.75	22	444444	000000	444	013
V	400	1.75	22	444444	000000	444	000
V	400	0.5	7	444444	000000	444	000
	1200	0.5	7			444	000
V	400	5.0	7	444443	000000	444	000
	1200	5.0	7			442	000
V	400	5.0	10	444444	000000	444	000
	1200	5.0	10			444	000

* Diluted 1:100 with 0.9% saline solution before use.

† In approximately half of each set of tubes in these two experiments the protein fraction was freed from bacteria by passage through a Seitz filter. In the remainder the fractions were treated in a 56 C water bath for 30 minutes. No significant difference in subsequent effects could be detected.

ponents tested included Fractions I, II, II+III, III-O, III-2, IV-1, IV-4, and V. In the third group thiourea was tested in the presence of purified amino acids, hydrolysates of casein, compounds which restore and others which cancel sulfhydryl group activity, and salts of certain heavy metals, individually or in combination (table 3).

The results of each experiment have been recorded on a scale of 0 to 4, with the latter representing maximal growth and the former no growth at all. Occasional differences in the luxuriance of growth are to be attributed to unavoidable nonuniformity in setting up the individual cultures.

TABLE 3.—*Summary of thiourea fungistatic activity following the addition of various substances.*

Substance	Concentration in medium (mg %)	Effects on thiourea activity
glycine (NH_2-free) l-(−)-tryptophane d-arginine-HCl dl-threonine dl-methionine dl-lysine-HCl dl-leucine dl-isoleucine dl-valine l-proline l-OH-proline dl glutamic acid dl aspartic acid dl alanine l-arginine-HCl l(−)-cystine l-lysine-HCl dl α alanine l(−)-histidine HCl d-glutamic acid	As high as or higher than those in which the individual amino acids are present in serum proteins.*	Thiourea still inhibits fungi.
Cysteine-HCl	100	Thiourea no longer inhibits fungi.
Casein hydrolysate	1000	Inhibitory even in absence of thiourea.
Casein hydrolysate +cysteine	1000 +100	Thiourea no longer inhibits fungi.
Tetra-decyl-Mercaptan†	10	
Sodium hyposulfite	10; 100	Thiourea no longer inhibits fungi.
Glutathione	23; 140	
Sodium thioglycollate	100	
Iodoacetic acid	0.00001; 0.000035	
Merthiolate	0.03 ; 0.5	
KCN	2.5 ; 25	Inhibitory even in absence of thiourea.
$ZnCl_2$	10; 50	
$HgCl_2$	‡	
$CuSO_4$ +glutathione	10 +140	Thiourea still inhibits fungi.
$CuSO_4$ +sodium thioglycollate	10 +100	

* Consult reference 3 in bibliography.
† Employed as an oily suspension in water.
‡ Still partially inhibitory in concentrations as low as 1:50,000.

RESULTS

It is immediately apparent from table 1 that the fraction or fractions of the serum or plasma proteins precipitated by a 33% solution of ammonium sulphate, i.e., the pseudo- and euglobulins, proved more effective than the albumin moiety in cancelling the activity of thiourea. In 8 experiments the proteins left in the supernatant fluid following the precipitation of the globulins, i.e., the albumin fraction, were added to the culture medium together with thiourea. The growth of *C. hominis* was nonetheless completely suppressed in 5. In the other 3 experiments only minimal reversal, if any, occurred. It is to be noted that even tripling the aliquot of albumin (first experiment in table 1) failed to block the fungistatic activity of thiourea. On the other hand, a combination of pseudo- and euglobulins, or of pseudoglobulins alone, added to 9 cc of dextrose-Sabouraud medium in amounts at least as great as those present in 1 cc of serum, reversed the thiourea blockage of fungus growth more definitely and more frequently than did the albumin

fraction. Thus, in 13 experiments in which these protein fractions were present together with thiourea, growth of *C. hominis* was observed in more than one-half of the plates. The effects of the euglobulin fraction alone in cancelling the thiourea activity were variable and less pronounced. Further attempts to clarify this point by using fractions obtained by precipitation with 40, 45, or 50% solutions of ammonium sulphate, respectively, failed to yield any more consistent results.[22]

The Cohn fractions on the other hand, with rare exception, failed to interfere with thiourea (table 2). This was true even though the individual proteins were present in concentrations as great as or greater than those resulting after the addition of serum or plasma itself. The sole exception (the second experiment with fraction IV-4) appears to be a sporadic and incomplete reversal. Occasional reversal of thiourea action was noted with unfiltered Cohn fractions.[22] However, since such plates had contaminant organisms, one cannot be certain that the reversal was not influenced by this factor.

Of the 23 amino acids tested, only cysteine, which alone contains a sulfhydryl group, cancelled the fungistatic activity of thiourea (table 3). A similar effect was noted in cultures to which tetradecyl mercaptan, sodium thioglycollate, glutathione, or sodium thiosulphate had been added. These compounds are known to replace or reconstitute the sulfhydryl (-SH) groupings, or provide a functionally acceptable substitute. The remainder of the experiments were inconclusive because the compounds, at the levels tested, themselves proved inhibitory for *C. hominis* even without thiourea. This was true of several commercial preparations of casein hydrolysate, iodoacetic acid, merthiolate, potassium cyanide, zinc chloride and mercury bichloride. It should be pointed out, however, that the addition of cysteine to medium which contained casein hydrolysate cancelled the inhibitory effects of the latter. This procedure was not tried with any of the other compounds. Finally, it is to be noted that copper sulphate interfered with the capacity of glutathione and sodium glycothiollate to cancel thiourea inhibition.

DISCUSSION

It is of interest that the suppression of fungus growth by thiourea, first described by Hachiya and Nishimura[6] and subsequently by Mayer,[7] is affected by certain protein components only. Albumin, for example, whether obtained in a relatively impure state as from ammonium sulphate treated serums or separated by the Cohn procedures as a highly purified chemical entity, fails to stop thiourea activity. This is not simply the result of employing in the culture medium less protein than would be present if serum itself had been used. Even with triple strength solutions of albumin, equivalent to adding serum containing 12 g per cent of protein, thiourea continued to suppress the growth of fungi.

This concept of specificity of interaction between thiourea and certain components of the serum or plasma proteins is further supported by the experiments with the globulins obtained from ammonium sulphate-treated serum. These fractions, whether used individually or in combination, reversed thiourea activity in about one-half of the experiments. In this series pseudoglobulin appeared more effective in this respect than did the euglobulins or the two

6. Hachiya and Nishimura. 1932. J. Phar. Soc. Jap. **52**: 91. (cited in Mayer, R. L., reference 7.)
7. Mayer, R. L. 1941, Recherches sur la chimiotherapie des Mycoses et de la Tuberculose. Revue Medicale de France, Nov.–Dec.

together. It should be noted, however, that in the studies with the individual fractions larger amounts of pseudo- than of euglobulins were employed. It may be, therefore, that the recorded differences are merely quantitative and not qualitative. Other factors may, however be responsible. For example, the results of ammonium sulphate separation of serum or plasma fractions are notoriously non-uniform. Furthermore, other evidence is now available that this method regularly leaves some globulins with the albumin portion.[3,5] Apparently this incomplete separation is not of sufficient import to prevent demonstration of a difference between albumins and globulins. It may, however, account for some of the discrepancies recorded in tables 1 and 2.

The studies with the Cohn fractions indicate that although cancellation of the fungistatic activity of thiourea would appear (from the ammonium sulphate fraction experiments) on the whole to be related to the globulins, it cannot be ascribed on the basis of our findings to any particular component of the non-albumin moiety isolated by the Cohn technique. The possibility cannot be excluded that the process of heating or of filtration before use altered the properties of the individual fractions.

The remaining data of this paper can be interpreted in the light of knowledge of agents which affect the sulfhydryl group. It would appear that thiourea, in common with certain other chemical agents and antibiotics, inactivates the sulfhydryl radical possibly by oxidation, alkylation, or mercaptide formation.[8–16] In this way it may suppress the growth of fungi. This is supported by the demonstration in our studies that cysteine, which contains the sulfhydryl group, cancels the fungistatic activity of thiourea, and that thioglycollate and glutathione act similarly. This view is also consistent with the observations of DeRitis, Scalfi, and co-workers, in which they demonstrated that the anti-bacterial effect of thiourea is blocked by serum cysteine, and thioglycollic acid.[17–20] It may well be that the occasional reversal

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of thiourea shown by ultrafiltrate[1] was due to the content of cysteine or similar compounds. The experiments with copper sulphate summarized in table 3 indicate that salts of this metal, like those of mercury, cancel the capacity of thioglycollate and glutathione to replace or regenerate sulfhydryl groups.

Even with the above data it is still not possible to explain why albumin fails to cancel the fungistatic activity of thiourea. In view of the essentially similar content of cysteine in the albumin and globulin moieties[3] both should be equally effective. Since this is not so, other factors must be operative. It is possible, for example, that in the albumin molecule, in contrast to its situation in the globulins, cysteine is inaccessible for replacement or reconstitution of the necessary sulfhydryl groups. This may depend on spatial relationships or may be conditioned by the presence of other electrical charges or amino acid groupings nearby.[3,21] Our attempts to investigate the last of these possibilities in cultures of *C. hominis* by adding in combination amino acids which alone have been shown to be ineffective have to date proven inconclusive.[22]

SUMMARY AND CONCLUSIONS

The reversal of thiourea activity by human serum or plasma appears to be related to the non-albumin fraction. Similar reversal was observed following the addition of substances which provide or reconstitute the sulfhydryl groupings. The constrasting effects of the albumin and non-albumin fractions of the serum or plasma proteins on thiourea activity have been discussed.

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INFECTIONS INDUCED IN MICE BY LOCAL APPLICATION OF STREPTOCOCCI AND PNEUMOCOCCI TO THE NASAL MUCOSA AND BY INTRAPULMONARY INSTILLATION

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Various workers have shown that particles containing bacteria are dispersed into the air by processes such as speaking, coughing, sneezing, bed making, and floor sweeping[1–6] and that these air-borne particles constitute a means whereby infections may be transmitted to individuals exposed to the atmosphere.[3–6] Some are large and settle rapidly, whereas others are fine and remain suspended in the air for long periods of time. The latter result principally from the rapid evaporation of large droplets of saliva or mucus emitted from the respiratory tract and are the "droplet nuclei" described by Wells.[6] Fine particles may also be redisperesd from the dry dust reservoir. Between the extremes of very fine particles and coarse ones there is a continuous gradation of particle diameters.

The relationship of diameter to the production of respiratory infection by bacteria-containing particles is a problem which requires clarification. Pertinent information on the subject has been provided by the experiments and calculations of several investigators[7–16]

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who have studied the role of particle size in the retention of chemical or inert aerosols in the respiratory tract. From their data the following generalizations can be made with regard to inhalation of particulate material, viz., a certain fraction of inhaled particles impinges and adheres at each level of the respiratory tract;[7] the number of particles retained depends upon their size,[7–12] rates and depths of inhalation,[10,11] nasal resistance to flow,[13,14] hygroscopicity,[10,15,16] as well as on the size of the species which is inhaling the material.[12] It is apparent from these considerations that in order to understand the role of particle size in production of respiratory infection one must first determine the susceptibility of the different portions of the respiratory tract on which inhaled particles will impinge. With this end in view, direct inoculation of the pathogens at two widely separated loci of the mouse respiratory tract was performed in the experiments reported here.

Intranasal inoculation of pathogens is a frequently employed bacteriological technique. Webster and Clow compared virulence of various strains of pneumococci by this and other routes.[17] Relatively large volumes of cultures were instilled and the amount of inoculum which actually remained on the nasal mucosa is not known. Schulz, Warren and Drinker[18] restricted the inoculum to the oral and nasal cavity by tying off the esophagus and trachea. By using minute volumes, we have found it possible to place the entire inoculum on the mouse nasal mucosa with reasonable certainty that it did not flow to other portions of the respiratory tract and thus were able to compare the median lethal doses of bacteria deposited onto the nasal mucosa with the comparable dose inoculated into the lungs. Studies of the pathology in mice dying after infection by these two routes were also made.

METHODS

Intranasal inoculation.—Mice were anesthetized with ether and held on a board as shown in plate 1, figure 1. The head was kept in position by means of a string which passed over the roof of the mouth behind the incisors. The inoculum was delivered by means of a "drod,"[19] a micrometer which displaces the barrel of a $\frac{1}{2}$ cc tuberculin syringe through small distances. The volume of the inoculum regularly employed was 0.0006 cc $\pm$ 20%. This variation in delivery has been checked several times by plate counts on a series of samples and by microchemical titration. The syringe was tipped with a blunted, smooth, 27 gauge needle which was inserted a distance of 3 mm into the right nasal cavity. In one series of experiments the needle was only touched to the right nares. The inoculum was delivered by turning the micrometer barrel of the drod through three scale divisions. The technique of inoculation is shown in plate 1, figure 1.

Intrapulmonary inoculation was carried out by the method of Jourdinais and Nungester[20] adapted to mice by Harford, Smith and Wood,[21] which consists in delivering the inoculum by means of a cannula introduced through mouth and larynx into the trachea. Visualization of the larynx was effected by transillumination of the

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neck according to the method suggested by Dr. J. M. Tobias of the Department of Physiology. The volume employed was 0.03 cc. Intravenous inoculation was performed in a lateral tail vein.

Inoculums.—Seven and one-half hour cultures of bacteria in serum-dextrose broth were prepared for inoculation by dilution with gelatin-Locke's solution in a series of 10-fold dilutions.[22]

Pathogens employed.—The hemolytic streptococcus Lancefield Group C was a highly mouse-virulent strain. It was originally obtained from infected cervical lymph nodes of a guinea pig and has been maintained by monthly mouse passage. The pneumococcus employed was the Type 1 A5 strain used in this laboratory for many years.[26] It has been maintained by rabbit and mouse passage and is highly virulent for these animals. The number of bacteria in the inoculums were determined by plating suitable dilutions in blood agar pour plates the day on which the culture was used.

Experimental animals.—The Germantown strain of Swiss mice was used for these experiments. All animals weighed between 10 and 20 g. They were kept in containers in groups of 5 after inoculations.

Autopsies.—The animals were observed for at least 10 days after inoculation although deaths were rarely observed after the 7th day. Autopsies were carried out on almost all animals which died. Tissues taken from several mice in each group for histological sections were fixed in Zenker-formol solution. Lungs were filled with the fixative by

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EXPLANATION OF PLATE 1

FIG. 1.—Method of intranasal deposition.

FIG. 2.—Dissection of cervical structures of a mouse which died following intranasal deposition of Streptococcus Group C. See tracing for identification of structures. 2×.

FIG. 3.—Dissection of cervical structures of a normal mouse. 2×.

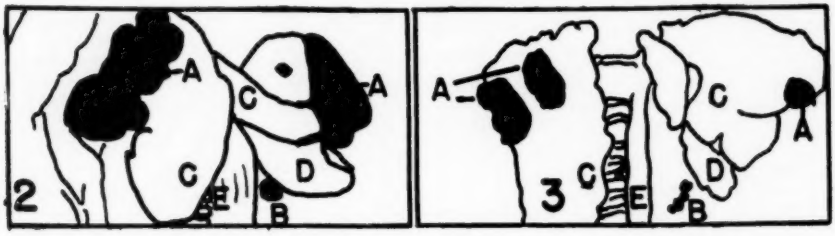
FIG. 4.—Photomicrograph of necrotic cervical lymph nodes of mouse dying from intranasally deposited streptococcus. The arrow points to a portion of one gland in which a few follicles and sinuses remain. 30×.

FIG. 5.—Parenchyma and hilum of lung of a mouse dying from intranasal streptococcal infection. Note perivascular and peribronchiolar edema, loss of architecture in hilar lymph nodes, and intact alveolar spaces. 10×.

FIG. 6.—Section from lung of mouse dying after intrapulmonary inoculation of streptococcus. Note extensive consolidation. 10×.

FIG. 7.—Same as figure 5 magnified 1000×. Note presence of bacteria in alveolar septa.

FIG. 8.—Same as figure 6 magnified 525×. Note bacteria and edema fluid free in the alveoli.



KEY TO PLATE 1

(Figures 2 and 3)

Lymph nodes are shown in black. A—superficial cervical lymph nodes. B—deep cervical lymph nodes. C—salivary gland. D—Sternocleidomastoid cut reflected anteriorly. E—Sternohyoid muscle and underlying trachea.

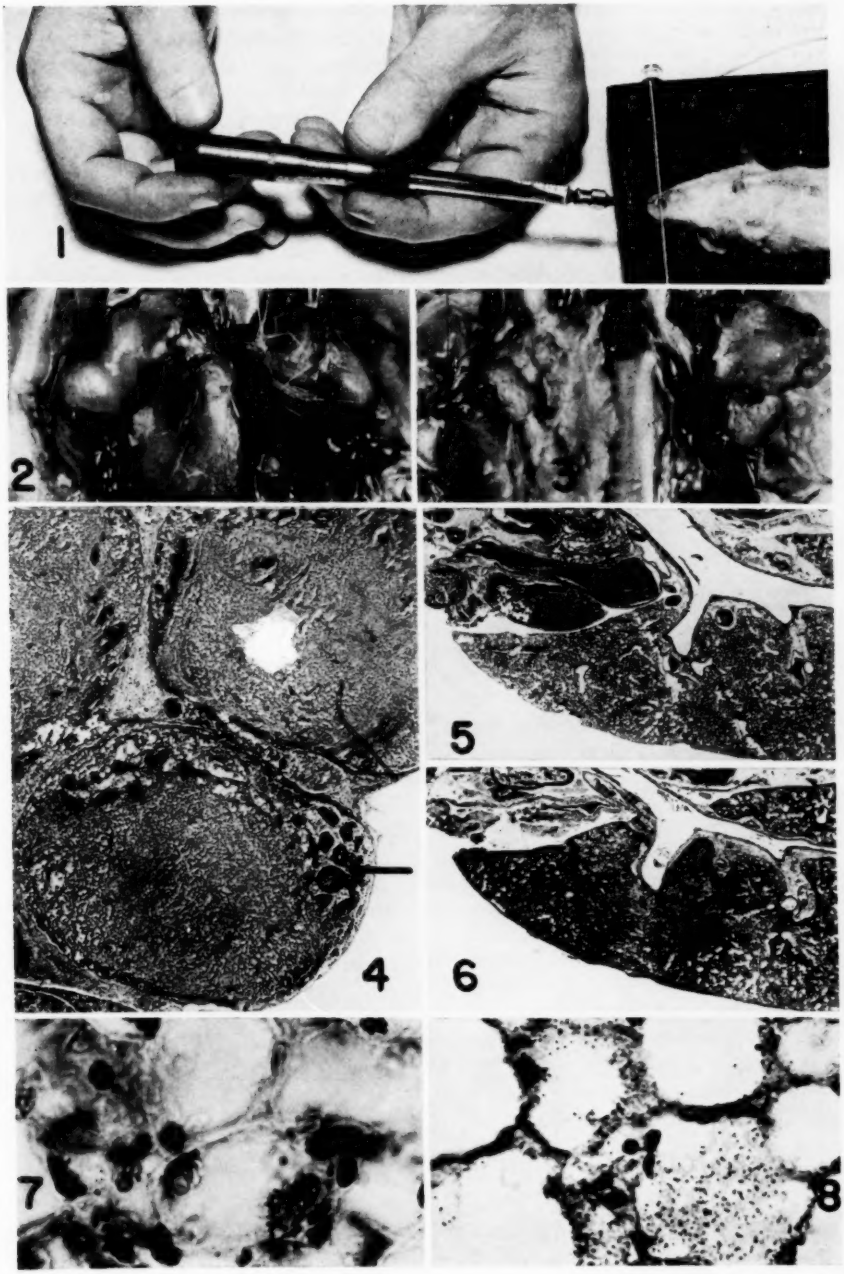


PLATE 1

the method of Loosli.[23] Sections were stained with the haemotoxylin-eosin-azure II stain and by the Wallace modification of the gram Weigert method.[24] In many cases the tissues were obtained from animals in extremis. Otherwise, they were obtained from animals which had not been dead longer than one hour.

EXPERIMENTAL

1. *Selection of a suitable volume of intranasal inoculum.*—In order to select a volume for intranasal inoculation ranging from 2×10^{-4} cc to 10^{-2} cc were instilled into the noses of several etherized mice. The animals were then sacrificed and the nose, digestive tracts, and upper respiratory tracts were examined for dye. It was found that 6×10^{-4} cc of dye inoculated with the needle placed 3 mm into the nares seemed to produce no staining of regions other than the nose and nasopharynx. Ten mice were then inoculated with this quantity of gentian violet. They were sacrifiecd with ether 10 minutes after the inoculation, dissected, and the parts examined for dye under the dissecting microscope. Table 1 summarizes the findings. In all cases dye was found in the right nasal cavity. Traces were present in the mouths of three animals and in the esophagus of one.* In no case was any dye found in the stomach, trachea, or lungs.

TABLE 1.—*Fate of 6×10^{-4} cc of gentian violet instilled into nares with a "drod."*

Mouse	Weight in g	Presence of gentian violet*			
		Nasal Cavity	Mouth	Trachea	Lung
1	16	+	–	–	–
2	17	+	–	–	–
3	27.5	+	–	–	–
4	26	+	–	–	–
5	23	+	trace	–	–
6	19	+	–	–	–
7	11	+	–	–	–
8	18	+	trace	–	–
9		+	trace	trace on epiglottis	
10	19	+	–	–	–

* Gentian violet was not present in the esophagus and stomach of any of the mice.

small enough not to be aspirated into the trachea or pharynx the following preliminary experiments were done. Volumes of saturated gentian violet solution in physiological salt solution

2. *Determination of the fate of particulate matter deposited on the nasal mucosa.*—Gentian violet is fixed in a short time by the nasal tissue with which it comes in contact. In order to determine the fate of particulate matter which is not fixed by the nasal mucosa, 6×10^{-4} cc of India ink were deposited in the noses of etherized mice by the above technique.

* The material in the mouths probably entered by way of two small apertures through the incisive canals which connect the oral and nasal cavities of mice.

TABLE 2.—*Fate of 6×10^{-4} cc of India ink particles instilled into nares of mice with a "drod."*

Mouse	Time sacrificed	Presence of carbon particles*					
		Right anterior nasal cavity	Left anterior nasal cavity	Post nasal cavity	Pharynx	Esophagus	Stomach
1 A	1 hour	+near roof	–	–	–	–	–
2 A	1 hour	+near roof	–	–	–	–	–
3 A	1 hour	+near roof	–	–	–	–	–
1 B	5 hours	+	–	–	–	–	–
2 B	5 hours	+	–	+	–	+	+
3 B	5 hours	+	–	+	–	–	–
1 C	24 hours	trace in anterior nares	–	–	–	–	–
2 C	24 hours	+	–	–	–	–	–
3 C	24 hours	trace in nares	–	–	–	–	–

* Carbon particles were not present in the trachea or lungs of any of the mice.

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Groups of three mice were sacrificed at periods ranging from 1 to 24 hours. They were dissected in the same manner as the mice inoculated with gentian violet and the organs were examined under the binocular microscope.

Table 2 summarizes the findings in this experiment. Animals sacrificed one hour after inoculation showed carbon in the right anterior nasal cavity only. Five hours after inoculation the ink was still found in the right anterior nasal cavity and in two of the mice, strings of blackened mucus were seen in the posterior nasal cavity over the soft palate. In one of them this material was found in the stomach contents. Mice sacrificed 24 hours after inoculation showed carbon black in the nares of one, and traces in the other two. In no case was any of the intransaally instilled material observed in the trachea or lungs.

3. *Mortality in mice after intranasal and intrapulmonary inoculation of Pneumococcus Type 1 and Streptococcus Group C.*—Groups of from 5 to 10 mice were inoculated by intranasal and intrapulmonary routes with different dilutions of Streptococcus Group C or Pneumococcus Type 1 suspended in gelatin-Locke's solution. Tables 3, 4, 5, and 6 summarize the mortalities observed in animals after inoculation. Figures 9, 10, 11, and 12 show the percent mortality plotted against the log of the dose administered by the two different routes and exhibit a considerable spread of doses at each percent mortality. There was a general increase in mortality with increasing dose in all of the experiments. The observed irregularities in these data would be smaller if all of the data were obtained from one experiment using the same group of mice and culture. It was more desirable from our point of view to obtain these data over a period of weeks in order to evaluate the irregularities which would occur in the repetition of the experiments, since we hoped to use the results to evaluate subsequent inhalation experiments.

TABLE 3.—*Mortality following intranasal deposition of beta hemolytic streptococcus Group C.*

Number of organisms per mouse	Mortality (days)							Total
	1	2	3	4	5	6	7*	
Experiment no. 1—11-9-46								
6×10^6	—	—	1	2	—	—	—	3/3
6×10^7	—	—	—	3	—	—	—	3/3
6×10^8	—	1	1	1	—	—	—	3/3
Experiment no. 2—11-16-46								
2.5×10	—	—	—	—	—	—	—	0/5
2.5×10^2	—	—	—	—	—	—	—	0/5
2.5×10^3	—	—	2	2	1	—	—	5/5
Experiment no. 3—11-23-46								
3.3×10^2	—	—	2	1	1	—	1	5/7
1.4×10^3	—	—	4	—	—	2	1	7/7
2.3×10^3	—	—	3	1	1	—	—	5/7
3.3×10^3	—	—	4	—	1	—	—	5/7
Experiment no. 4—12-9-46								
2.9	—	—	—	—	1	—	—	1/10
2.9×10	—	—	1	—	1	—	—	2/10
2.9×10^2	—	—	6	—	1	—	—	7/10
2.9×10^3	—	1	7	2	—	—	—	10/10
2.9×10^4	—	1	5	2	1	—	—	9/10
						Total mice		102

* None of the mice died after 7 days.

The LD_{50}'s were calculated from the data by the method of Bliss.[25] The fiducial limits of each LD_{50} were com-

25. Bliss, C. I. 1938, The determination of the dosage mortality curve from small numbers. Quart. J. Pharm. & Pharmacol. **11:** 192–216.

TABLE 4.—*Mortality following intrapulmonary inoculation of beta hemolytic streptococcus Group C.*

Number of organisms per mouse	Mortality (days)										Total
	1	2	3	4	5	6	7	8	9	10	
				Experiment no. 1—10-30-46							
5.3×10^{2}	—	1	1	2	1						5/5
2.7×10^{3}	—	—	2	1							3/4
5.3×10^{3}	—	—	4	1							5/5
2.7×10^{4}	—	—	2	3							5/5
				Experiment no. 2—11-11-46							
5.9											0/5
1.2×10^{1}											0/5
5.9×10^{1}											0/5
1.2×10^{2}											0/5
				Experiment no. 3—11-23-46							
1.4×10^{2}	—	—	—	—	—	1	—	—	—	1 strep.	1/5
1.4×10^{3}	—	—	—	2	1	—	1	—	—	—	4/5
1.4×10^{4}	—	1	4	—	—	—	—	—	—	—	5/5
				Experiment no. 4—11-30-46							
3.8	—	—	—	—	—	—	—	—	—	—	0/5
7.6	—	1									1/5
3.8×10	—	—	—	—	—	—	—	—	—	—	0/5
7.6×10	—	—	—	1	—	—	—	—	—	—	1/5
3.8×10^{2}	—	—	1	—	—	—	—	—	—	—	1/5
7.6×10^{2}	—	—	—	—	—	—	—	—	—	—	1/5
3.8×10^{3}	—	1	1	1	1	—	—	—	—	—	4/5
							Total mice				89

puted by this method in order to indicate the extreme values expected in 19 out of 20 similar experiments. The fiducial limits depend upon the scatter, slope, and position of the points in figures 9–12. Any LD_{50} which falls outside of the fiducial limits of a determination is significantly different from that of the determination. Table 7 summarizes the calculated LD_{50}'s and fiducial limits. The LD_{50} for intranasal streptococcus falls between 180 and 410 bacteria per mouse, and the intrapulmonary value lies between 340 to 1000 bacteria per mouse. The LD_{50} of intranasally deposited pneumococcus lies between 300 and 160,000 bacteria per mouse, while intrapulmonarily inoculated pneumococci have an LD_{50} between 22,000 and 130,000 bacteria per mouse. The LD_{50} for intranasal deposition of Streptococcus Group C is slightly lower than the LD_{50} for intrapulmonary instillation. Thus, the animals were somewhat more susceptible to the intranasal infection than they were to intrapulmonary infection by the methods employed. The LD_{50}'s observed with intranasal and

TABLE 5.—*Mortality following intranasal deposition of Pneumococcus Type 1.*

Dilution	Dose—organisms per mouse	Mortality (days)				Total time observed (days)	Mortality
		1	2	3	4*		
			Experiment no. 1—1-8-47				
10^{-4}	10^{1}	—	—	—	—	12	0/5
10^{-3}	10^{2}	—	1	2	—	12	3/5
Culture	10^{9}	—	3	2	—	3	5/5
			Experiment no. 2—1-14-47				
10^{-5}	2.8	—	—	—	—	21	0/10
10^{-4}	2.8×10	—	—	—	—	21	0/10
10^{-3}	2.8×10^{2}	—	—	—	—	21	0/10
10^{-2}	2.8×10^{3}	—	1	4	—	21	5/10
10^{-1}	2.8×10^{4}	—	—	6	2	21	8/10
			Experiment no. 3—1-18-47				
10^{-3}	3.1×10^{2}	—	—	3	—	18	3/10
10^{-2}	3.1×10^{3}	—	—	3	2	18	5/10
10^{-1}	3.1×10^{4}	—	—	5	—	18	5/10
Culture	3.1×10^{5}	—	1	5	1	18	7/10
					Total mice		105

* None of the mice died after 4 days.

TABLE 6.—*Mortality following intrapulmonary inoculations of Pneumococcus Type 1.*

Dilution	Dose—organisms per mouse	Mortality (days) 1	2	3	4*	Total time observed (days)	Mortality
		Experiment no. 1—1-18-47					
10^{-3}	1×10^{3}	—	—	—	—	18	0/5
10^{-4}	1×10^{2}	—	—	—	1	18	1/5
10^{-3}	1×10^{4}	—	—	—	1	18	1/3
10^{-2}	1×10^{5}	—	—	—	1	18	1/5
		Experiment no. 2—1-27-47					
10^{-4}	4.6×10^{2}	—	—	—	—	25	0/5
10^{-3}	4.6×10^{3}	—	—	—	—	25	0/5
10^{-2}	4.6×10^{4}	—	1	1	—	25	2/5
10^{-1}	4.6×10^{5}	—	2	1	—	25	3/5
Culture	4.6×10^{6}	—	2	1	—	25	3/5
		Experiment no. 3—2-22-47					
10^{-4}	4	—	—	—	—	7	0/5
10^{-3}	4×10	—	—	—	—	7	0/5
10^{-2}	4×10^{2}	—	—	—	—	7	0/5
10^{-1}	4×10^{3}	—	1	—	1	7	2/5
Culture	4×10^{4}	—	1	—	3	7	4/5
		Experiment no. 4—3-1-47					
10^{-4}	2×10^{2}	—	—	—	—	7	0/3
10^{-3}	2×10^{3}	—	—	—	—	7	0/3
10^{-2}	2×10^{4}	—	—	—	—	7	0/5
10^{-1}	2×10^{5}	—	—	4	1	4	5/5
Culture	2×10^{6}	—	—	1	3	7	4/5
					Total mice		89

* None of the mice died after 4 days.

intrapulmonary Type 1 pneumococcus were considerably higher than those observed with the streptococcus and were not significantly different from each other within the error of this experiment. However, the wide variations observed in the experiments with pneumococcus permit only a rough comparison of the intrapulmonary and intranasal infections produced in the manner described in this report. Undoubtedly the use of a larger number of animals in the experiments would decrease this variation.

4. *The effect of inoculating Streptococcus Group C into the esophagus of mice.*—The foregoing experiments raise the possibility of infection by bacteria which may have been swept back into the mouth by ciliary action and swallowed after intranasal instillation. A priori it would seem that the mouse esophagus with its cornified squamous epithelium is not a likely site for bacterial invasion of the host. In order to answer the question of esophageal infection two groups of 20 mice each were inoculated with 6×10^{-4} cc of Streptococcus Group C cultures containing a total of 3×10^{5} bacteria. One group was inoculated intranasally by the method described; the other group was inoculated by passing the needle of the "drod" through the mouth into the esophagus of the etherized mouse. In order to do this the animal was held down as for intranasal instillation and a blunted needle with a shaft 1 cm long was slid back into the mouth until the base of the needle was arrested by the molars. The needle

TABLE 7.—*LD_{50}'s with fiducial limits following infections with Streptococcus Group C and Pneumococcus Type 1.*

Organism	Site of deposition	Mean LD_{50}	Fiducial limits	Number of mice
Streptococcus C	Nose	2.7×10^{3}	$(1.8\text{–}4.1)\times10^{3}$	102
Streptococcus C	Lung	5.6×10^{2}	$(3.4\text{–}10)\times10^{2}$	89
Pneumococcus	Nose	6.5×10^{3}	$(0.3\text{–}160)\times10^{3}$	105
Pneumococcus	Lung	5.5×10^{4}	$(2.2\text{–}13)\times10^{4}$	89

slid easily into the esophagus. Its position was ascertained by sacrificing and dissecting several mice while the needle was in situ.

All of the intranasally inoculated mice died from 2 to 6 days after inoculation with characteristic pathological changes to be described in a following section, while none of the intraesophageally inoculated mice succumbed. The same experiment was repeated with Type 1 pneumococcus with similar results. Therefore, it is concluded that swallowed streptococci and pneumococci do not give rise to a lethal infection.

5. *The effect of varying the method of intranasal deposition of Streptococcus Group C on the outcome of infections in mice.*—In order to evaluate the role of trauma to the nasal mucosa concomitant with introduction of the blunted needle into the nose and its subsequent influence on the outcome of infections, groups of 15 or 20 etherized mice were inoculated intranasally with 6×10^{-4} cc of a Streptococcus Group C culture containing from 4.6×10^{2} to 4.6×10^{5} bacteria by the following two methods.

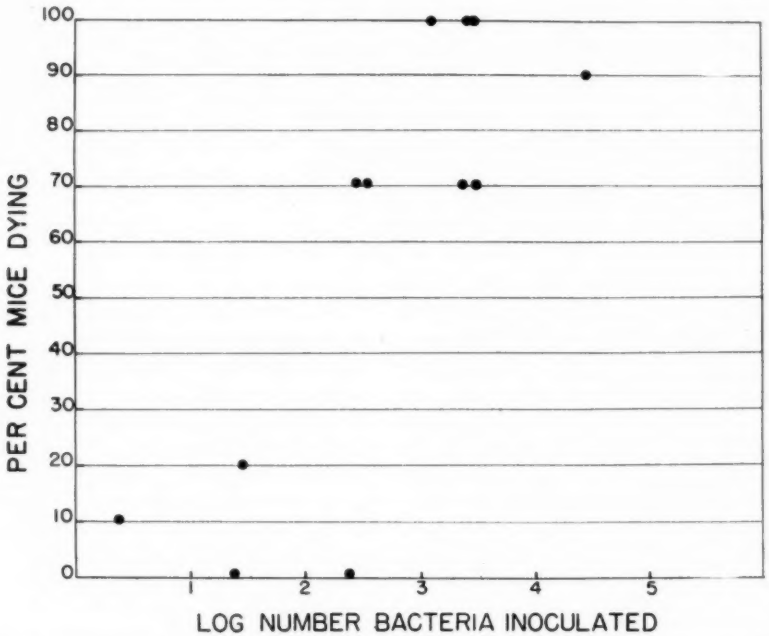

FIG. 9.—Mortality following intranasal deposition of varying doses of Streptococcus Group C.

Series I: The blunted 27 gauge hypodermic needle was placed 3 mm into the right nares, and the inoculum was introduced. This corresponded to the method employed in experiments 3 and 4. It will be referred to as intranasal deposition or inoculation in the following discussion.

Series II: The inoculum was transferred from the tip of the needle onto the right external nares from which place the mouse drew it into the nose by

inspiration. The needle made no direct contact with the mouse. This will be referred to as supranasal deposition.

Table 8 shows that the intranasal mortality is higher than the supranasal for each dose. This difference is in part explained by the loss of part of the inoculum which spreads on the nose after supranasal deposition, and perhaps partly on the basis of increased susceptibility to the infection following damage to the nasal mucosa when the cannula is inserted. The pathology resulting from these two methods of deposition onto the nasal mucosa was identical.

The LD_{50}, approximately 4.6×10^4 bacteria per mouse, observed in the intranasally instilled group in this experiment is considerably higher than that observed in the earlier experiments exhibited in table 7, line 1, which had been carried out several months previously. Changes in virulence of the laboratory culture or in the mice used could account for this difference. It is obvious that frequent checks of the dosage mortality relationship must be made in the course of any group of experiments of this sort which extend over a period of days or weeks.

6. *Pathological changes occurring in mice after inoculation of Streptococcus Group C and Pneumococcus Type 1 by various routes.*—*Lymph nodes.*—Striking changes were seen grossly in the cervical lymph nodes of mice which received intranasal inoculations of Streptococcus Group C. These glands were visibly swollen and could be felt through the skin as hard subcutaneous swellings. The dissected nodes which were enlarged many times above normal (plate 1, fig. 2 and 3) appeared yellow, opaque, and ruptured with slight pressure extruding a cheesy yellow mass. Gram stains of smears of this material showed it to be full of gram-positive streptococci. In many animals lymphorhexis had occurred, causing a cellulitis in the head, cervical, and chest regions. Mesentric and inguinal lymph nodes from the same animals were not grossly involved although occasional histological sections revealed small abscesses which were undoubtedly secondary to pyemia in these mice. Plate 1, figure 4 shows a section of a necrotic cervical node in which the architecture is destroyed everywhere but in a corner of the node.

TABLE 8.—*Effects of intranasal and supranasal inoculation of various doses of Streptococcus Group C in mice.*

Dose—bacteria per mouse	Mortality—intranasal inoculation	Mortality—supranasal inoculation
4.6×10^5	10/15	4/15
4.6×10^4	7/15	5/15
4.6×10^3	3/15	1/15
4.6×10^2	2/20	1/20

Table 9 shows a comparison of the incidence of necrotic cervical lymphadenopathy occurring in all animals dying after intranasal, intrapulmonary, and intravenous inoculation of Streptococcus Group C and Pneumococcus Type 1. All but two mice which died after receiving intranasal inoculations of Streptococcus Group C exhibited this

TABLE 9.—*Occurrence of purulent cervical lymphadenitis in mice after inoculation of Streptococcus Group C and Type 1 Pneumococcus by different routes.*

Organisms	Route of inoculation	Number of animals dying and autopsied	Number showing purulent cervical lymphadenitis
Streptococcus C	Intranasal	161	159
Streptococcus C	Intrapulmonary	42	1
Streptococcus C	Intravenous	10	1
Type 1 Pneumococcus	Intranasal	105	0
Type 1 Pneumococcus	Intrapulmonary	86	0

pathological change. The two in which it did not occur were dead in less than 48 hours after inoculation. Their cervical nodes were markedly injected. It is believed that these two animals died from the infection before the caseation had time to occur. A marked septicemia accompanied all fatal infections.

Lung changes.—Pulmonary infections occurred in most of the animals regardless of the route of inoculation with streptococci or pneumococci.

Intranasal deposition: Grossly the lungs from intranasally inoculated animals were either a normal pink or showed varying degrees of red mottling

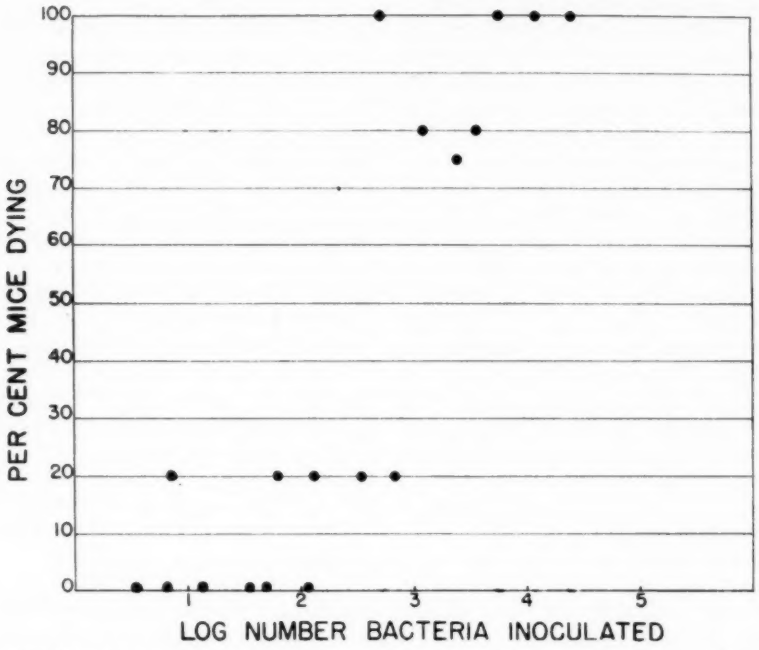

FIG. 10.—Mortality following intrapulmonary inoculation of varying doses of Streptococcus Group C.

Only 1 in 42 mice dying after intrapulmonary instillation of hemolytic streptococci showed enlarged caseous cervical nodes, although all had severe pneumonia. In the intravenous series of inoculations, cervical lymphonecrosis occurred in 1 out of 10 mice. This pathological change was not seen in animals dying from Type 1 pneumococcus infection initiated by any route, and the cervical nodes of these animals were usually small and markedly injected.

and wetness. Sections of lungs of animals inoculated intranasally with Streptococcus Group C revealed marked mediastinal, perivascular, and peribronchiolar edema and in many cases exhibited large numbers of microorganisms in this fluid. Bacteria were also seen in blood vessels in the interalveolar septa. The hilar lymph nodes contained many streptococci and were partially or entirely necrotic. Involvement of the thymus was frequent. Bacteria were not found in the trachea, bronchi, bronchi-

oles, or alveoli. Intraalveolar hemorrhage or edema was observed occasionally in small isolated areas or dependent portions of the lung. The blood vessels were congested. This terminal picture could be the result of local bacterial invasion of tissues from the blood stream, but the present data are inadequate to be sure of this point (plate 1, fig. 5 and 7).

ing proportions. Large numbers of bacteria were present in the alveoli (plate 1, fig. 8) and in exudates occurring in the bronchi and trachea.

While histological study of pulmonary changes in mice infected by the intrapulmonary route with Pneumococcus Type 1 was less extensive, lung sections from two mice dying showed abundant intraalveolar growth of bacteria. A

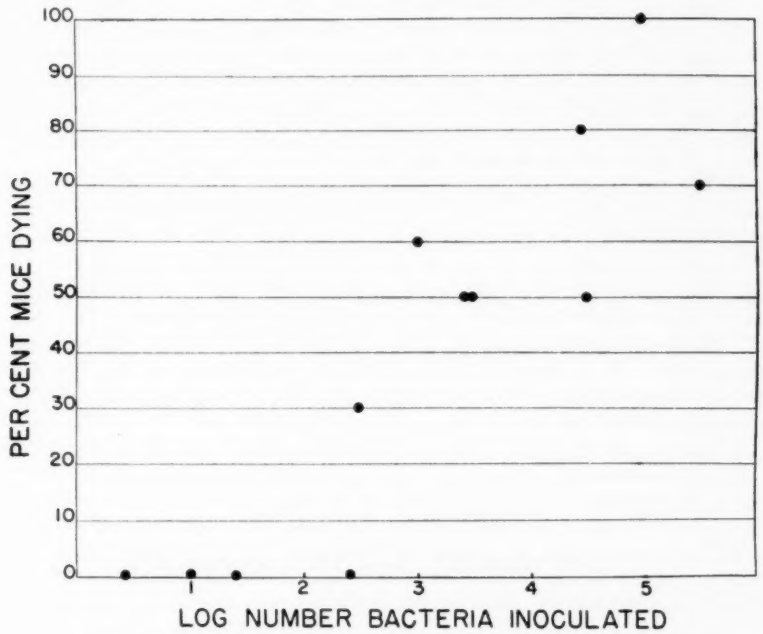

FIG. 11.—Mortality following intranasal deposition of Pneumococcus Type 1.

Intrapulmonary inoculation: Lung sections from animals which died after intrapulmonary inoculation with Streptococcus Group C showed, in addition to the above findings, localized necrotic areas of various sizes and stages of development in all lobes (plate 1, fig. 7). The cellular inflammatory reaction was slight and the lesions in all stages of development consisted almost entirely of bacteria, edema, and debris in varyseries of sections obtained from mice sacrificed at various times after intranasal instillation of pneumococci revealed bacteria in the septal blood vessels of the lungs but none in the air spaces.

7. *Histological examination of heads and tissues of mice at varying times after intranasal instillation of Pneumococcus Type 1.*—In the case of the intranasal streptococcal infections the constant

and specific involvement of the regional, cervical lymphatics suggested a role of these glands in the pathogenesis of this infection. This was not obvious, however, with the pneumococcus, since the cervical lymph nodes showed no marked pathological change and active growth of pneumococci could not be demonstrated in them. Thus, there was no apparent focus at which these bacteria multiplied before an overwhelming infection occurred. The following experiments were undertaken to determine whether or not a focus of multiplication existed at any point between the site of deposition and the cervical lymph nodes.

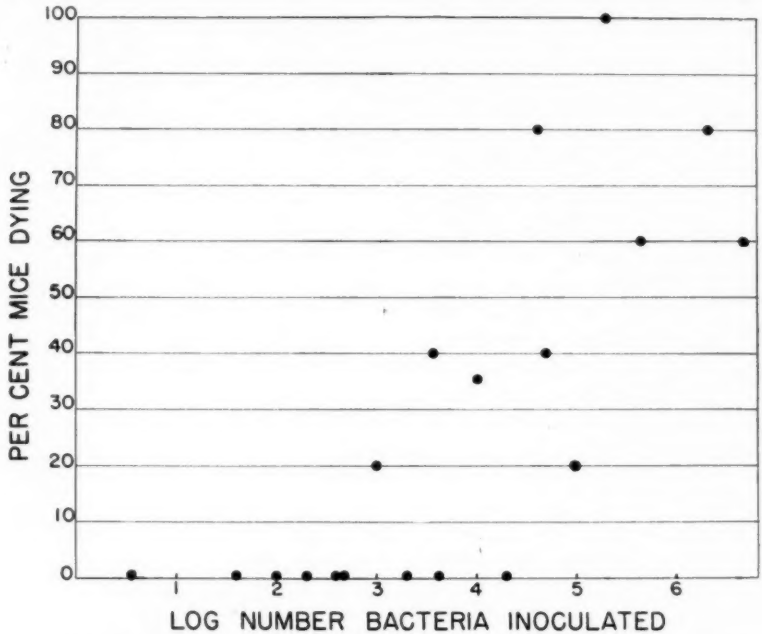

FIG. 12.—Mortality following intrapulmonary inoculation of varying doses of Pneumococcus Type 1.

Groups of mice were inoculated intranasally with Pneumococcus Type 1 by the method described above. Each mouse received 3×10^4 bacteria. Animals were sacrificed with intravenous sodium pentobarbital at various times between 1 and 48 hours after inoculation. The heads were fixed in 10% formalin after stripping off the skin, and histological sections from each head at several transverse planes were stained by the Wallace modification of the gram Weigert technique. Sections from other tissues were prepared by the method described earlier in this report. At the time the mice were sacrificed blood cultures were made by streaking two drops on blood agar plates which were then incubated at 37 C for 24 hours and examined. A total of 220 sections were examined from various levels of the heads of 20 mice sacrificed up to $13\frac{1}{2}$ hours after instillation. Head sections from animals infected for longer periods were not examined since bacteremia had

occurred in most of them by this time.

The nasal mucosa of the mouse is typical, ciliated respiratory epithelium. Bacteria were not demonstrable on the nasal mucosa in any of these sections nor was any focus of active bacterial growth found at any point between the nasal mucosa and cervical lymph nodes up to and during the earlier periods in which bacteremia was seen microscopically. Blood cultures were positive for pneumococci as early as 10½ hours after intranasal deposition, and in many instances microorganisms were detected microscopically in the blood vessels 16 hours after inoculation.

DISCUSSION

These experiments clearly demonstrate that fatal infections can be produced in mice by certain pathogenic bacteria deposited at two widely separated loci in the respiratory tract, namely, the nasal mucosa and the lung. Furthermore, the pathological changes seen in mice dying after Streptococcal Group C inoculation by the two routes were distinctly different and enable one to differentiate the site of infection in almost every fatal case. This may be a useful observation with regard to inhalation experiments in which it should be possible to determine whether the lethal, inhaled dose of streptococci acted by way of the nasal mucosa, lung, or both. Experiments of this sort are now in progress.

One must be cautious in inferring a separate pathogenesis for these two distinctly different types of streptococcal infections since all of the animals were autopsied terminally. Robertson, Coggeshall and Terrell[26] have pointed out the importance of examining groups of infected animals at repeated intervals between the time of infection and the time of death in studies of pathogenesis. However, a striking aspect of the present study is that the two types of pathological change were sufficiently different to remain distinct after septicemia and overwhelming infection had occurred. The marked, constant, cervical lymphatic necrosis contrasted with the relatively mild involvement or absence of involvement of other regional lymph nodes in animals infected intranasally with the streptococcus is strong evidence that the infection travels through the nasal mucosa to the regional cervical lymph nodes by way of the lymphatics of the head.

It is of interest in this regard that the intranasally inoculated pneumococcus produced a fatal systemic infection despite the absence of localization in the cervical lymph nodes or elsewhere. Thus, while localization of Streptococcus Group C in the lymph nodes of the neck appears to precede systemic infection, the data secured in the present study of intranasally initiated infection do not exclude the possibility that some streptococci may gain entrance into the blood stream without preliminary localization in the cervical lymphatics. Both lymphatic infection and direct invasion of the blood stream have been observed by other investigators. Schulz, Warren and Drinker[18] have described the cervical lymphatic route for large molecules and pathogenic bacteria placed in the noses of dogs and rabbits. Large amounts of material deposited in the nose and mouth were collected from the cannulated cervical lymphatic duct after a short time. Tuttle and Cannon[27] have demonstrated the rapid passage

26. Robertson, O. H., Coggeshall, L. T. and Terrell, E. E. 1933, Experimental pneumococcus lobar pneumonia in the dog. III. Pathogenesis. J. Clin. Investigation **12**: 467–493.

27. Tuttle, W. M. and Cannon, P. R. 1935, The passage of bacteria from the lungs into thd blood stream. J. Infect. Dis. **56**: 31–37.

of *Streptococcus hemolyticus* through the intact dog's lung into the blood capillaries, whereas the thoracic duct lymph remained sterile up to 60 minutes after inoculation. Loosli[28] has demonstrated the rapid passage of Pneumococcus Type 1 into the pulmonary lymphatics of dogs after intrabronchial inoculation. Direct invasion of the blood stream was not observed. Burt, Tuttle and Cannon[29] found that this organism could be recovered from the blood stream of rabbits within a few minutes after intratracheal introduction. Thus, it appears that direct infection of the blood stream or lymphatics may occur depending upon the species of animal and the bacteria employed.

The cervical lymphatic infection which we have found in mice is not unlike that which was observed clinically as a complication of tonsillitis before the advent of the sulfonamides and penicillin, although it should be pointed out that the human cervical infection is not regularly succeeded by bacteremia as is the mouse infection. It bears some resemblance to the endemic, chronic cervical lymphatic infections which have been described in guinea pig colonies.[30] Indeed, the streptococcus culture used in these experiments was obtained originally from a guinea pig in which infection had occurred spontaneously, and we have found that it has retained its ability to produce a chronic cervical lymphadenitis in this species. The process described here differs from that occurring in guinea pigs in that it is not readily transmitted from infected to uninfected mice in the same container and it is in all cases an acute disease. These characteristics may account at least in part for the fact that streptococcal cervical lymphadenitis is not seen to occur spontaneously in laboratory mouse colonies.

The role of the anesthetic on these infections has not been evaluated here. Stillman and Schulz[31] have demonstrated that etherization increases the susceptibility of mice to the pneumococcus. It would be desirable to obtain data on the effects of intranasal and intrapulmonary inoculation of the bacteria in unanesthetized mice. The technical difficulties of these procedures, however, are obvious.

The Streptococcus Group C seems to be an ideal microorganism for the study of particle size in its relation to airborne infection in mice, for the reasons that the distinctive pathological changes which we have observed enable one to differentiate a nasal from a pulmonary infection in almost every fatal outcome and the end points in dosage mortality titrations performed over a period of weeks are fairly sharp with this microorganism.

These experiments clearly demonstrate the necessity of controlling the possible role of intranasal infection in studies of the histogenesis of experimental pneumonia, a point which has been overlooked by some workers.[32,33]

28. Loosli, C. G. Personal communication.
29. Burt, K. L., Tuttle, W. M. and Cannon, P. R. 1932, Studies of the local immunization of the lungs of rabbits with pneumococcus Type 1. Proc. Soc. Exper. Biol. & Med. **30:** 1138–1139.
30. Moen, J. K. 1936, Tissue culture studies in bacterial hypersensitivity. II. Reactions of tissues from guinea pigs infected with Group C streptococci. J. Exper. Med. **64:** 355–368.
31. Stillman, E. G. and Schulz, R. Z. 1940, Susceptibility of mice to intranasal instillation of various types of pneumococci. J. Infect. Dis. **66:** 174–177.
32. Young, G. A., Jr., Zelle, M. R. and Lincoln, R. E. 1946, Respiratory pathogenicity of *B. anthracis* spores. I. Methods of study and observations on pathogenesis. J. Infect. Dis. **79:** 233–246.
33. Hoyle, L. and Orr, J. W. 1945, The histogenesis of experimental pneumonia in mice. J. Path. **57:** 441–449.

SUMMARY AND CONCLUSIONS

Streptococcus Group C and Pneumococcus Type 1 were deposited by means of a micrometer syringe device on the nasal mucosa, in the lung, and in the esophagus of mice. Volumes were employed which permitted the original inoculum to be deposited and confined to the above mentioned sites without flowing into neighboring structures.

1. Beta hemolytic Streptococci Group C were slightly more lethal to etherized mice when deposited on the nasal mucosa than when inoculated into the lungs. The LD_{50}'s of Pneumococcus Type 1 by both routes in etherized mice were the same within the limits of error of the experiment but were higher than the LD_{50}'s of the streptococcus.

2. The variability of the dosage mortality data obtained with the pneumococcus indicates that titrations of mice with doses of this organism will not provide an accurate end point for quantitative comparisons of groups of data obtained by methods comparable to those described here. End points obtained with Group C Streptococcus are satisfactory for this purpose.

3. Deposition of streptococcal cultures on the undamaged nasal mucosa causes a lethal infection in mice.

4. Interesophageal inoculation failed to cause infection.

5. All but 2 of 161 animals dying after intranasal inoculation with Streptococcus Group C developed enlarged, purulent lymph nodes, whereas mesentric and inguinal nodes showed little or no involvement. Only 2 mice out of 52 autopsied after fatal intrapulmonary instillation and intravenous inoculation developed similar cervical lymphatic pathology.

None of the mice dying after pneumococcal inoculations by any route developed purulent cervical lymph nodes. Pneumococci were not observed on the nasal mucosa or in any of the tissues of the head and neck at any time between one hour after intranasal deposition and the onset of bacteremia 10 to 16 hours later.

6. The lung infections in mice which died after intranasal inoculation was of an interstitial type, whereas in mice dying after intrapulmonary inoculation abundant bacterial growth occurred in the air spaces.

The bearing of these findings on experimental air-borne infections is discussed.

ISOLATION OF EASTERN EQUINE ENCEPHALOMYELITIS VIRUS FROM NATURALLY INFECTED MONKEY (*MACACUS PHILIPPINENSIS*)

HARVEY R. LIVESAY*

A native monkey was bought by an army officer from a Filipino in the vicinity of Manila about December 25, 1946. It was then given to a female civilian living in the Manila Engineers Compound (H Street), where it was kept. On April 14, 1947 it became sluggish and would not eat. On April 15 it had difficulty in walking and on the following day was brought to the Veterinary Section of the 3rd Medical General Laboratory with a flaccid paralysis of the left hind leg. It was caged, observed and sent to the virus section on April 17. The next day the paralysis involved the right hind leg. It was sacrificed for virus study on April 18, 1947. This monkey had been in contact with two other monkeys in the Manila compound and had also contacted humans living in the area. No symptoms of any central nervous system disease were observed in any contact.

Reaction in animals

Brain and cord suspensions were injected into monkeys, mice, rats and guinea pigs (fig. 1). The suspensions were first injected intracerebrally into 4 mice on April 19. One mouse died of trauma, one mouse was paralyzed on the 4th day and two mice showed no symptoms. A second passage, which was bacteriologically sterile, was made into 4 mice from the paralyzed animal on April 23. Two of these second passage mice showed paralysis and died on the 2nd and 4th days. One mouse was paralyzed on the second day and it was used for the 3rd passage. The 4th passage was inoculated into rats, which responded similarly.

Suspensions from the original monkey were also inoculated intracerebrally into two monkeys (20351 and 20352) on April 24. These animals showed no clinical evidence of disease by May 2. At this time two more monkeys (20340 and 20350) were inoculated from the original material. Of the latter two monkeys, one (20340) was dead on the 5th day without clinical symptoms, but it showed an encephalomyelitis on section (table 1). The other monkey (20350) developed a high fever on May 10 with flaccid paralysis of the hind legs and one fore leg on May 13 (fig. 2). This monkey was sacrificed May 13. Tissue from this monkey was used for making the second monkey passages. The second passage monkeys (20358 and 20359) developed a more acute infection with no evidence of paralysis of the hind legs (fig. 1 and fig. 2). There was, however, paralysis of the neck muscles.

Virus identification

Cross protection tests done at the 3rd Medical General Laboratory with a limited number of viruses indicated that the virus was probably the eastern equine encephalomyelitis strain. It was specifically identified as the eastern strain of equine encephalomyelitis virus by Smadel[1] of the Virus and Rickettsial Disease Laboratory of the Army Medical Department Research and Graduate School, Washington, D. C. He summarized his findings as follows:

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Captains Reuben Zucker, Clarence A. McWhorter, Paul C. Stonesifer, Dr. Enrique Salafranca, and Dr. Lourdes Espiritu of the virus section of the 3rd Medical General Laboratory aided in this project, and their help and cooperation are gratefully acknowledged.

* U. S. Army, 3rd Medical General Laboratory, APO 900, c/o Postmaster, San Francisco, California.

1. Smadel, James E. 1947, Personal communication.

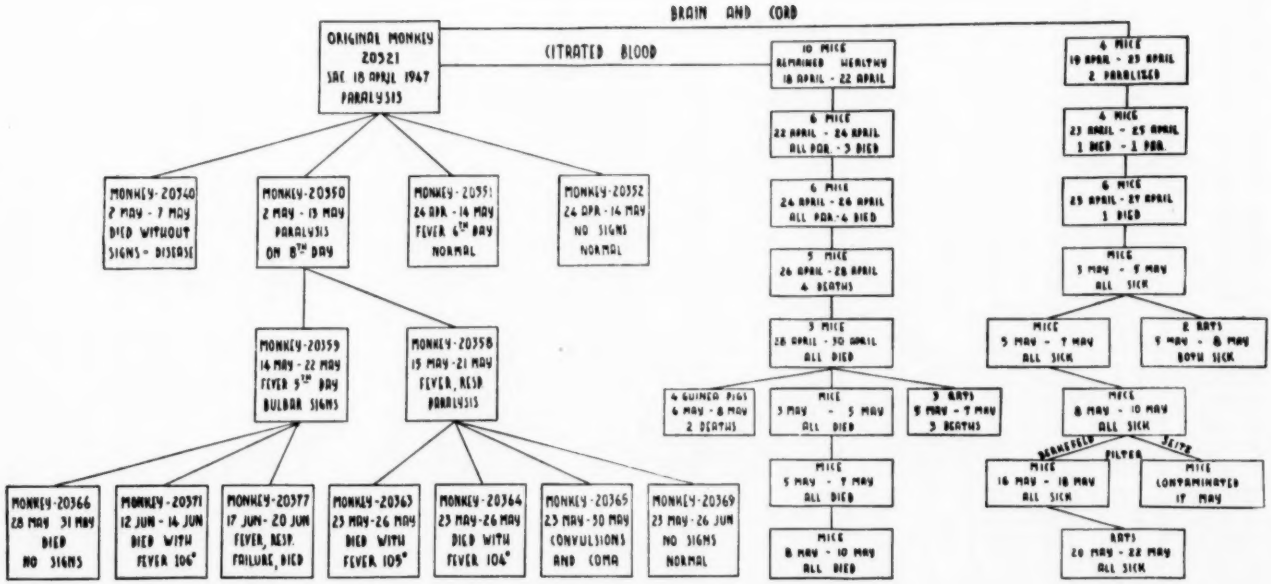
BRAIN AND CORD
ORIGINAL MONKEY 20321 SAC 18 APRIL 1947 PARALYSIS
CITRATED BLOOD
10 MICE REMAINED HEALTHY 18 APRIL - 22 APRIL
6 MICE 22 APRIL - 24 APRIL ALL PDR - 3 DIED
6 MICE 24 APRIL - 26 APRIL ALL PDR - 6 DIED
5 MICE 26 APRIL - 28 APRIL 4 DEATHS
3 MICE 28 APRIL - 30 APRIL ALL DIED
4 GUINEA PIGS 6 MAY - 8 MAY 2 DEATHS
MICE 3 MAY - 5 MAY ALL DIED
5 RATS 5 MAY - 7 MAY 3 DEATHS
MICE 5 MAY - 7 MAY ALL DIED
MICE 8 MAY - 10 MAY ALL DIED
4 MICE 19 APR - 23 APRIL 2 PARALYSED
4 MICE 23 APRIL - 25 APRIL 1 DIED - 1 PDR.
6 MICE 25 APRIL - 27 APRIL 1 DIED
MICE 3 MAY - 5 MAY ALL SICK
MICE 5 MAY - 7 MAY ALL SICK
2 RATS 5 MAY - 8 MAY BOTH SICK
MICE 8 MAY - 10 MAY ALL SICK
BENEFIELD
FILTER
SEITZ
MICE 16 MAY - 18 MAY ALL SICK
MICE CONTAMINATED 17 MAY
RATS 20 MAY - 22 MAY ALL SICK
MONKEY-20340 2 MAY - 7 MAY DIED WITHOUT SIGNS - DISEASE
MONKEY-20350 2 MAY - 13 MAY PARALYSIS ON 8TH DAY
MONKEY-20351 24 APR - 14 MAY FEVER 6TH DAY NORMAL
MONKEY-20352 24 APR - 14 MAY NO SIGNS NORMAL
MONKEY-20359 14 MAY - 22 MAY FEVER 5TH DAY BULBAR SIGNS
MONKEY-20358 15 MAY - 21 MAY FEVER, RESP. PARALYSIS
MONKEY-20366 28 MAY 31 MAY DIED NO SIGNS
MONKEY-20371 12 JUN - 14 JUN DIED WITH FEVER 104°
MONKEY-20377 17 JUN - 20 JUN FEVER, RESP. FAILURE, DIED
MONKEY-20363 23 MAY - 26 MAY DIED WITH FEVER 105°
MONKEY-20364 23 MAY - 26 MAY DIED WITH FEVER 104°
MONKEY-20365 23 MAY - 30 MAY CONVULSIONS AND COMA
MONKEY-20369 23 MAY - 26 JUN NO SIGNS NORMAL

FIG. 1.—Results of injections of brain and cord suspensions.

A summary of the findings of this laboratory pertinent to the problem of eastern equine encephalomyelitis virus in the Philippines is as follows:

a. Eastern equine encephalomyelitis virus has been isolated from monkey brains representing the second and third monkey passages from the original rhesus animal.

b. No agent could be isolated from the original material (Monkey #20321) in two attempts.

TABLE 1.—*Data on monkeys during three passages of the encephalomyelitis virus.*

Monkey number	Inoculum	Route*	Observations	Pathological diagnosis
			First passage:	
20352	Cord Brain	icer iper	No symptoms noted up to 20th day after inoculation. No rise in temperature. Sacrificed.	Normal spinal cord and brain
20340	Cord Brain	icer iper & inas	Found dead 5 days after inoculation. No signs of illness.	Encephalomyelitis
20351	Cord Brain	icer iper & inas	No signs or symptoms noted except slight rise of temperature (103 F) on 6th day. Sacrificed on 20th day.	Normal heart, brain and cord
20350	Cord Brain	icer iper & inas	Rise in temperature (105.8 F) with tremors of the right leg noted on the 8th day. Paralysis of right on 10th day with weakness of the left leg and tremors of the upper extremities and right upper extremity on the 11th day. Sacrificed 11th day.	
			Second passage:	
20359	Cord Brain	icer iper & inas	Temperature of 104.6 F, inability to swallow, salivation, lacrimation, tremors of the head, symptoms of respiratory failure, definite muscle tenderness, positive Kernig's all noted on the 8th day. No paralysis.	Possible encephalitis
20358	Cord Brain	icer iper	Temperature of 105.6 F, tremors of left leg and spasms of muscles noted on the 5th day. Breathing was noisy and rapid. Tracheotomy performed on the 6th day because of respiratory distress. Death. Autopsy 6th day.	
			Third passage:	
20365	Brain	iper & inas	No significant rise in temperature. Rapid respiration, muscle pains and convulsions developed on the 6th day. Kernig's sign was positive. The following day the animal was semicomatous, with temperature of 94 F, nystagmus, loss of muscle tone in the right arm and failing respiration. Died 7th day.	Encephalomyelitis
20369	Brains	iper & inas	No symptoms or signs of illness noted within an observation period of 30 days.	
20363	Cord	icer	Temperature of 105 F developed on the second day. Found dead on the 3rd day without any other signs of illness noted.	
20364	Cord	icer	Temperature of 103.8 F noted on the 2nd day. Found dead on the 3rd day. No other signs of illness noted.	

* icer—intracerebral.
iper—intraperitoneal.
inas—intranasal.

c. A virus identical with eastern equine encephalomyelitis was also obtained from mouse and guinea pig material representing a passage (number unknown) from the original monkey.

No virus of this type had been used in the 3rd Medical General Laboratory prior to May 22, 1947, when a sealed lyophilized ampule was opened. This virus was used in cross protection tests in an effort to identify the etiological factor responsible for the clinical and pathologic evidence of encephalomyelitis in the inoculated animals.

Other evidence of the disease

The eastern strain of equine encephalomyelitis had never been isolated nor had the disease been reported as a clinical entity in the Philippine Islands prior to the work described in this report.[2] Since the virus was found, the work of Mace et al[3] using neutralization tests on horse serums further confirms evidence of the presence of the virus

2. Ferriols, V. and Gomez, A. K. 1948, Personal communication.
3. Mace, D. L. and Cortez, F. S. 1949, Evidence of the unsuspected presence of equine encephalomyelitis virus in Philippine animals. In press.

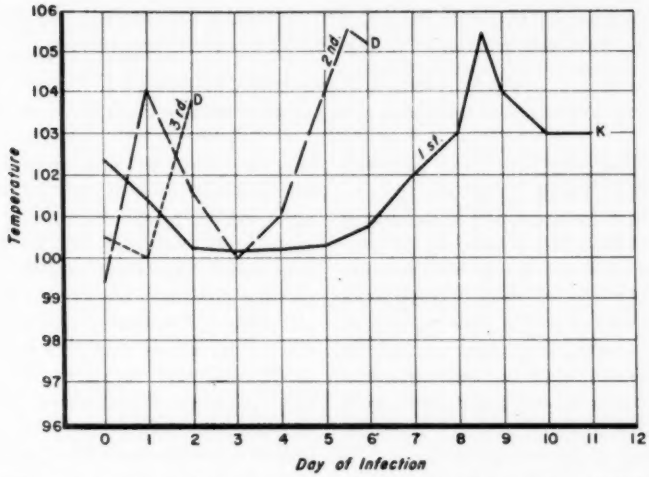

FIG. 2.—Temperature curves of three passages of the virus into three monkeys (20350, 20358 and 20364, respectively).

in the Islands. It is also known that during the Japanese occupation army horses south of Manila near Batangas had a disease called forage poisoning. Whether this was equine encephalomyelitis is not known, but is presumptive evidence of the disease.

SUMMARY

The clinical observations on the disease in the Philippine monkey are similar to known poliomyelitis, in that paralysis of the flaccid type is common in the hind legs. In the more acute form of the disease the flaccid type paralysis may be absent. The incubation period is usually shorter. (Compare figures 1, 2 and 3.)

The eastern type of equine encephalomyelitis virus has been isolated and identified as the causative agent of a natural disease in the native Philippine monkey (*Macacus philippinensis*).

A STUDY OF THE MODE OF ACTION OF SUBSTANCES WHICH ENHANCE BACTERIAL INVASIVENESS

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Since Nungester et al[1] first reported that gastric mucin enhances the invasiveness of bacteria, it has become a common laboratory procedure to employ suspensions of mucin for this purpose. In recent years many investigators have used mucin with a wide variety of bacterial species, but there is still no general agreement as to the mechanism by which mucin and substances with similar pathogenizing activity alter the invasiveness of microorganisms.

Nungester et al[2] were of the opinion that the viscosity and cohesive properties of mucin were important indices of its effectiveness. They stated that mucin does not interfere with phagocytosis. Tunnicliff,[3] however, reported that gastric and salivary mucin produce a coating on bacteria which prevents their staining vitally and inhibits their phagocytosis. This view does not agree with the data of Anderson and Oag,[4] who studied the pathogenizing activity of different chemical fractions of mucin and concluded that "the action of mucin does not appear to be of a physical nature." Supporting this opinion, Gould and King[5] fractionated mucin preparations and obtained approximately a 20-fold concentration of the "virulence enhancement" factor without any great increase in viscosity. They suggested that mucin contains a substance comparable to the bacterial aggressins, although Miller and Castles[6] had concluded previously that this was not the mode of action since, when mucin is used to suspend organisms for subcutaneous injection, no aggressin-like action or enhancement of invasiveness follows.

Many substances other than mucin have this capacity to increase the invasiveness of bacteria. Olitzki et al[7] investigated the pathogenizing effect of a wide variety of carbohydrates. Levans produced by *Bacillus subtilis* and *Aerobacter levanicum* and a dextran from *Leuconostoc mesenteroides* proved to be comparable to mucin in pathogenizing activity. Since Anderson and Oag[4] concluded that the active principle of mucin is found in the protein fraction, Olitzki tested a levan which had been synthesized in vitro by the action of cell-free *Aerobacter* enzyme. This synthetic levan was also effective as a pathogenizing agent, thus proving that such activity is not confined to proteins but is also an intrinsic property of some polysaccharides.

It is obvious that an understanding of the mechanism by which such substances as bacterial polysaccharides enhance bacterial invasiveness would be

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1. Nungester, W. J., Wolf, A. A. and Jourdonais, L. F. 1932, Proc. Soc. Exper. Biol. & Med. **30:** 120–121.
2. Nungester, W. J., Jourdonais, L. F. and Wolf, A. A. 1936, J. Infect. Dis. **59:** 11–21.
3. Tunnicliff, R. 1940, J. Infect. Dis. **66:** 189–191.
4. Anderson, C. G. and Oag, R. K. 1939, Brit. J. Exper. Path. **20:** 25–32.
5. Gould, J. C. and King, H. K. 1947, Biochem. J. **41:** xxi.
6. Miller, C. P. and Castles, R. 1936, J. Infect. Dis. **58:** 263–279.
7. Olitzki, L., Shelubsky, M. and Hestrin, S. 1946, Proc. Soc. Exper. Biol. & Med. **63:** 493–496.

extremely significant in the study of natural infectious mechanisms. Witebsky et al[8] and Beeson and Goebel,[9] among others, have shown that an extremely close chemical and immunological relationship exists between some blood group substances and the capsular polysaccharides of certain pneumococci. Morgan and King[10] found that blood group substances function as resistance lowering factors, and Friedemann et al[11] showed that they also increase capillary permeability.

It seemed possible that the property of increasing capillary permeability might be a characteristic not only of blood group substances but of other pathogenizers as well. The work reported here was undertaken to determine whether or not mucin and some other known pathogenizing agents increase capillary permeability.

MATERIALS AND METHODS

The methods used in this work for determining the effect of substances on capillary permeability have been reviewed in detail by Friedemann.[12] Groups of guinea pigs receive intramuscular injections of a standard dose of tetanus toxin immediately following the intravenous administration of serial dilutions of antitoxin. The toxin is mixed with the substance under test, and a control series receives toxin in saline. If the test substance increases capillary permeability, a higher dilution of antitoxin in the circulation suffices to protect the animals against tetanus. In the rabbit skin test, the animal receives a standard dose of diphtheria antitoxin intravenously. Immediately following this, serial dilutions of diphtheria toxin mixed with the test substance are injected intracutaneously down one side of the shaved abdomen. The same dilutions of toxin in saline are injected on the other side. If capillary permeability is increased, the skin reactions are diminished. In both tests an increase in capillary permeability is indicated by an enhanced neutralizing effect of antitoxin.

Wilson's type 1701-W mucin was used. Preparations of 1 to 5% were made by grinding in a mortar, suspending in distilled water, autoclaving at 15 pounds for 15 to 20 minutes, and adjusting the p_H to 6.5 to 7.0 with sterile sodium hydroxide.

Purified pneumococcal capsular polysaccharides, types I and VII, were prepared according to the methods of Heidelberger et al.[13] The type I polysaccharide (hereinafter referred to as S-I) was extremely antigenic when tested in mice. The S-VII was not tested for antigenicity.

Suspensions of organisms grown on Bacto dextrose-starch agar were used to prepare the polysaccharides of *B. subtilis* and *Aerobacter cloacae*. Both suspensions were heated at 70 C for 30 minutes, after which the organisms were removed by centrifugation. The supernatant from the *B. subtilis* suspension was Seitz filtered. The polysaccharides were prepared by concentrating the supernatants and precipitating repeatedly with alcohol.

The polysaccharide of *L. mesenteroides* was prepared in a similar manner, except that the culture was grown in broth containing 5% sucrose. Incubation was at 22 to 25 C for one week.

The toxins and antitoxins of both tetanus and diphtheria were very generously supplied by the Lederle Laboratories, Inc. Lot numbers and unitage are designated in the tables of results.

Guinea pigs weighing approximately 250 g received antitoxin in quantities of 0.5 ml injected intracardially under light ether anesthesia. This method of injection has been used on more than 150 animals without difficulty or accidental loss of animals. Immediately following administration of antitoxin, the toxin, diluted in the test substance, was injected into the muscle of the right rear leg.

EXPERIMENTAL

In addition to groups of animals receiving tetanus toxin diluted in the test materials, two control groups were used. One control group received toxin diluted in saline, and the other received toxin diluted in 1/5000 histamine diphos-

8. Witebsky, E., Neter, E. and Sobotka, H. 1935, J. Exper. Med. **61:** 703–715.
9. Beeson, P. B. and Goebel, W. 1939, J. Exper. Med. **70:** 239–247.
10. Morgan, W. T. J. and King, H. K. 1943, Biochem. J. **37:** 640–651.
11. Friedemann, U., Traub, F. B. and Langstadt, D. 1947, Proc. Soc. Exper. Biol. & Med. **64:** 434–436.
12. Friedemann, U. 1947, Bact. Rev. **11:** 275–302.
13. Heidelberger, M., Kendall, F. E. and Scherp, H. W. 1936, J. Exper. Med. **64:** 559.

TABLE 1.—*The effect of various pathogenizers on capillary permeability of guinea pigs.*

Units of antitoxin per 0.5 ml	Toxin diluents							
	Saline	Histamine	1% mucin	5% mucin	S-I 4.0 mg/ml	S-I 0.8 mg/ml	*Aerobacter* carbohydrate	*B. subtilis* carbohydrate
200	L	O	O	O	O	O	O	O
100	L	O	O	O	O	O	O	O
50	S	O	O	O	O	O	O	O
25	S	O	O	O	O	O	O	O
12.5	S	O	O	O	L	L	L	O
6.2	D	O	L	L	L	S	S	S
3.1	D	L	L	O	S	D	L	L
1.5	D	L	L	L	S	S	S	S

Antitoxin (lot no. 6799) injected intracardially in a volume of 0.5 ml. Tetanal toxin (lot no. 1632) injected intramuscularly in a constant volume of 0.2 ml.
O—No symptoms of tetanus.
L—Local tetanus, ranging from the slightest evidence of stiffness to paralysis of the injected leg.
S—Severe tetanus, ranging from paralysis of both rear legs to complete incapacitation.
D—Died of tetanus.

phate. The latter has been shown by Friedemann to increase capillary permeability in dilutions as high as 1/100,000.

As may be seen in table 1, all of the substances tested significantly increase the availability of antitoxin for the neutralization of tetanus toxin. S-I is effective even when the individual guinea pig dose is only 0.16 mg. We have not generally interpreted results in terms of Friedemann's Ai^0/Ai' values. If this is done, however, we find that the substances tested here are comparable in action to peptone broth, blood group AB substance and other preparations tested by Friedemann. The fact that variation in concentration of mucin and S-I does not appreciably alter their effect on capillary permeability is in agreement with Friedemann's[12,14] conclusion that a substance either does or does not increase permeability, i.e., there is apparently no direct correlation between the effect of an active substance and its concentration above an effective minimum.

In obtaining the results shown in table 1, toxin and antitoxin dilutions were used in such concentrations that few of the animals would receive fatal doses. In this case, those animals showing no visible symptoms of tetanus are considered to be most significant. In table 2 are shown the results obtained when the relative quantity of toxin was increased to the point where most of the animals received normally fatal doses. In this case, survival is taken as the criterion of effectiveness of test substance.

All of the preparations shown in tables 1 and 2 have also been tested by Friedemann's rabbit skin test method. Strangely enough, none of the bacterial polysaccharides increase the capillary permeability of rabbits. Of interest also is the fact that while some lots of mucin appear to have such activity, other lots have little or no significant effect on permeability. Some typical results of such tests are shown in table 3. Friedemann has made no reference to such difference in results to be obtained when identical substances are tested by these

TABLE 2.—*The effect of the carbohydrates of* Leuconostoc mesenteroides *and pneumococcal S-VII on capillary permeability of guinea pigs.*

Units of antitoxin per 0.5ml	Toxin diluents		
	Saline	*Leuconostoc* carbohydrate	S-VII
40	S	L	L
20	D	L	L
10	D	S	L
5	D	S	L
2.5	D	S	L
1.2	D	D	S

Antitoxin (lot no. 6799) injected intracardially in a volume of 0.5 ml. Tetanal toxin (lot no. 1632) injected intramuscularly in a constant volume of 0.2 ml.
L, S, D—Symbols as in table 1.

14. Friedemann, U., Traub, F. B. and Langstadt, D. 1946, J. Immunol. **54**: 197–206.

two methods, although Traub et al[15] did report that the potentiation of tetanus toxin by broth and serum is marked in mice and guinea pigs but absent in cats and rabbits. We were led to consider the possibility of a species difference in response to the test substances. Although the rat is relatively resistant to tetanus toxin, tests were made of histamine, mucin and S-I in rats, using a procedure identical with that employed in guinea pigs. As might be expected, results of preliminary experiments were less consistent than were those routinely obtained with the more susceptible guinea pigs. However, when a sufficient number of animals was employed, results similar to those shown in table 4 were obtained. Without attempting to obtain the Ai^0/Ai' ratio, the difference in the number of animals showing any observable symptoms of tetanus in the different groups appears to be significant.

All of the materials which we have tested and found to increase capillary permeability are known to increase the invasiveness of certain bacteria. However, because of the difference in effect on rabbit capillary permeability of the three lots of mucin shown in table 3, the pathogenizing activity of similar preparations was tested in mice. It was found that lot B decreased the MLD of a culture of *Staphylococcus aureus* approximately 100-fold, while lots A and C had no effect whatever on the invasiveness of the same culture. These preliminary results suggest that the pathogenizing potency of mucin may be correlated directly with its capacity to increase capillary permeability.

TABLE 3.—*The effect of different lots of mucin and of certain bacterial carbohydrates on capillary permeability of rabbits.*

Toxin diluents	Dilutions of toxin				
	1:100	1:200	1:400	1:800	1:1600
Saline	++++	+++	+++	++	+
Mucin lot A	+++	++	++	+	±
Mucin lot B	++	+	±	−	−
Mucin lot C	+++	+++	++	++	+
Saline	++++	+++	+++	+	±
S-I	+++	++	++	±	−
Leuconostoc carbohydrate	++++	+++	++	+	−
B. subtilis carbohydrate	+++*	++*	++	±	−

Diphtheria antitoxin (lot no. 1520–24) injected intravenously. Toxin (lot no. 2562) diluted in test substance and injected intracutaneously.
+ = Relative degree of erythema.
* Necrosis present.

TABLE 4.—*The effect of histamine, S-I and mucin on capillary permeability of rats.*

Units of antitoxin per 0.5 ml	Toxin diluents											
	Saline			Histamine			S-I			Mucin		
160	O	O	S	O	O	O	O	O	O	O	O	O
80	O	O	S	O	O	O	O	O	S	O	O	L
40	S	S	S	O	O	S	O	O	O	O	O	S
20	S	S	S	O	O	L	O	O	O	O	O	S
10	L	S	S	O	O	O	O	O	O	O	S	S
Summary of animals	11/15			2/15			1/15			5/15		

Antitoxin (lot no. 6799) injected intracardially in a volume of 0.5 ml. Tetanal toxin (lot no. 1632) injected intramuscularly in a constant volume of 0.2 ml.
O, L, S—Symbols as in table 1.
Summary—Number of animals showing symptoms/number of animals in series.

DISCUSSION

Since our experimental work was

15. Traub, F. B., Hollander, A. and Friedemann, U. 1946, J. Bact. **52**: 169–177.

completed, Olitzki[16] has published a review of mucin as a resistance-lowering substance. He concludes that "most of the observations made with mucin in intraperitoneal infections can be ascribed to a basic phenomenon: the coating effect of mucin on bacteria." We believe that it is possible to interpret many data, including our own, in a different way.

There are adequate data to support the suggestion that the pathogenizing effect of mucin and certain polysaccharides may depend ultimately on their capacity to induce a particular sequence of events associated with inflammation. Ercoli et al[17] and Gould and King[5] have discussed the aggressin-like character of the action of gastric mucin. The fact that all of the pathogenizing substances which we have tested increase capillary permeability suggests that this aggressin-like action may be characteristic of most pathogenizing substances and that it may be due to an innate capacity to stimulate the inflammatory response with its attendant increase in capillary permeability.

Superficially, the suggestion that pathogenizers enhance invasiveness by inciting inflammation may appear to be paradoxical in view of Menkin's extensive studies which have led to the general concept that inflammation is a defensive mechanism which, among other things, enables an animal to resist bacterial invasion. It should be noted, however, that studies of the inflammatory reactions have usually been done in noncavitary tissues, in which case inflammation acts to localize the irritating agent under conditions inimical to bacterial multiplication. In contrast to this, pathogenizing agents are effective only when injected into serous cavities. This may be due to the fact that in serous sacs, during inflammation, fibrin is deposited peripherally on the serous surfaces after an initial accumulation of serous fluid within the cavity. Thus, in a nonimmune animal, the bacteria find an excellent growth medium within the peritoneal cavity. At the same time, the fibrin "envelope," once it is formed, might well inhibit the migration of leucocytes to the freely multiplying bacteria within the fluid of the cavity. Oerskov[18] found, in fact, that when various bacteria are injected with mucin into the peritoneal cavity, migration of fresh leucocytes to that area is checked within a few hours. McLeod[19] and Olitzki et al,[20] among others, have noted that frequently when either saline or mucin suspensions of bacteria are injected into the peritoneal cavities of mice, they appear rapidly in the blood stream but disappear after about two hours. This disappearance from the blood very probably coincides with the formation of the fibrin barrier which not only prevents the movement of bacteria from the focus of infection into the circulation but also restricts the movement of leucocytes into the peritoneal cavity. If the animal is immune, however, its serum and serous fluid would contain antibodies which might be the determining factor in the ultimate course of the infection.

Olitzki[16] has concluded that an effect on barrier permeability, as suggested by Miller and Castles,[6] plays no part in the

16. Olitzki, L. 1948, Bact. Rev. **12:** 149–172.
17. Ercoli, N., Lewis, M. N. and Harker, E. 1945, Proc. Soc. Exper. Biol. & Med. **59:** 273–278.
18. Oerskov, J. 1940, Report of Proceedings, Third International Congress of Microbiol. **771:** New York.
19. McLeod, Charlotte. 1941, Am. J. Hyg. Sec. B. **34:** 51–63.
20. Olitzki, L., Koch, P. K. and Shelubsky, M. 1947, Exper. Med. & Surg. **5:** 206–218.

mechanism of the action of pathogenizers. His basis for this view, however, appears to be the fact that bacteria injected intraperitoneally appear very promptly in the circulation with or without the presence of mucin within the peritoneal cavity. The effect of mucin on the barrier between the peritoneal cavity and the circulation may be one of altering, not the ability of microorganisms to pass through these tissues, but, by virtue of an inflammatory action, their ability to multiply unrestrictedly until their numbers and toxic products can overwhelm the host. It is generally agreed that, whatever the basic mechanism of mucin's action may be, one of its chief functions appears to be to create a focus of infection which eventually pours an overwhelming number of bacteria into the circulation. Indeed, it seems quite possible that death of an animal with a mucin-enhanced infection may be due to an accumulation within its body of approximately the same quantities of "toxic substances" which would suffice to kill the animal if injected as a single dose without mucin.

The experiments of Ercoli et al[17] lend considerable support to the conception that mucin affects some phase of the inflammatory response. Not only did the injection of various bacteria with mucin fail to elicit a characteristic leucocytic response in the peritoneal cavity, but even the leucocytic reaction of aleuronat was inhibited by the simultaneous injection of mucin. It is significant, furthermore, that the "virulence-enhancing effect of mucin can be neutralized by leucocytosis previously induced with aleuronat." It appears most probable then that mucin stimulates the formation of some barrier to leucocytic migration although it possesses no antagonistic effect towards leucocytes. It seems unlikely that the coating effect of mucin on bacteria plays any part in these phenomena since a previously induced leucocytosis neutralizes the effect of mucin.

It is known that the viscosity of a pathogenizing preparation sometimes influences its activity; that the concentration of active material is directly related, within limits, to effectiveness;[7] that active substances have a high molecular weight; and that an increase in volume of inoculum results in a disproportionate increase in pathogenizing effect.[21] These observations indicate that a pathogenizer must not be removed too quickly from the peritoneal cavity by absorption. If removal is too rapid, as is true of histamine and similar substances which increase capillary permeability but have no pathogenizing effect, there is no enhancement of invasiveness. According to Olitzki's review, this was demonstrated directly by Sindbjerg-Hansen,[22] who found that mice recovered from an otherwise fatal injection of bacteria and mucin if the peritoneal cavity were washed out with sterile saline within four hours after inoculation. The necessity for slow absorption is further indicated by the complete ineffectiveness of pathogenizers injected intravenously, although, if the pathogenizing agent is injected intraperitoneally, bacteria injected intravenously acquire an increased pathogenicity. This is to be expected, since the movement of bacteria from the circulation to the peritoneal cavity would be relatively unrestricted during the first hours after infection.

21. McLeod, Charlotte. 1941, Am. J. Hyg. Sec. B. **34:** 41–50.
22. Sindbjerg-Hansen, V. 1943. Einar Munksgaard, Kopenhagen. Studier over den infektious fremmende virkning af mucin paa svagt patogene bakterier. Cited in Olitzki, L. 1948, Bact. Rev. **12:** 149–172.

The inactivity of mucin when injected intravenously is difficult to reconcile with Olitzki's[16] belief that the coating effect of mucin on bacteria is the key to the mechanism of its action. So, also, is the report of Olitzki et al[23] that mucin and other pathogenizers inhibit antibody absorption by bacteria. In the first place, they report that pectin, as well as mucin, dextran and levan, coat the bacteria and block their specific surface receptors. If pathogenizers function in this manner, then pectin should have such activity. Olitzki et al,[7] however, found that it does not. Furthermore, if pathogenizers inhibit antibody absorption by coating the bacteria, it is difficult to understand the close correlation which exists between in vitro antibody titers and mouse protection tests in which mucin is used, or, indeed, why it is possible to demonstrate any immunity using the mucin technique.

As is well known, different lots of gastric mucin may differ in their pathogenizing effect. It is also known that such samples vary considerably in their content of blood group substances which Morgan and King[10] have shown to enhance bacterial invasiveness. Commercial gastric mucin preparations are chemically complex, and it seems quite likely that variation in pathogenizing activity may be due to differences in the quantity of inflammatory materials present, whether blood group substances or other constituents. It seems quite possible, furthermore, that kaolin, which Olitzki et al[7] have shown enhances the activity of pathogenizers, does so by virtue of a supplementary irritating action on tissues, rather than by an effect on the coating of bacteria.

It is not intended to imply that pathogenizing activity is due exclusively to the capacity to incite an inflammatory response. Obviously, ability to produce inflammation is not restricted to substances which enhance bacterial invasiveness. The problem of why substances with almost identical chemical and physical properties vary so markedly in their pathogenizing activity remains, even though our results suggest that an inflammatory mechanism is involved. Olitzki et al[7] have suggested "that the pathogenizing activity of a polysaccharide is a function of patterns which are specific to the colloidal state." This observation and our data, as well as the pathological course of events which appears to be characteristic of infections enhanced by pathogenizing substances, lead us to suggest that a hypersensitive type of tissue damage may be involved. Investigations based on this hypothesis are in progress.

SUMMARY

Preparations of gastric mucin and certain bacterial polysaccharides which have in common the capacity to enhance invasiveness of microorganisms have been shown to increase capillary permeability in the guinea pig. Of the substances tested, only gastric mucin has a comparable effect in rabbits. The possibility that this capacity to increase capillary permeability is causally related to the action of pathogenizing agents has been discussed. Objections have been raised to the view that mucin enhances invasiveness by virtue of its coating effect on microorganisms.

23. Olitzki, L., Shelubsky, M. and Efrati, E. 1947, Proc. Soc. Exper. Biol. & Med. **64**: 258–259.

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